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STUDIES ON POLYCHROME METHYLENE BLUE

II. Acid Oxidation Methods of Polychroming

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ABSTRACT.—**The action of $K_2Cr_2O_7$, Ag_2O, $KMnO_4$, HgO and $NaIO_3$ in polychroming methylene blue is explored. The last two have no action in neutral or acid methylene blue solutions. With the other three reagents the amount of polychroming, as measured by the shift in the absorption spectrum, is roughly proportional to the amount of oxidant used. Various lots of methylene blue produce similar products with similar proportions of $K_2Cr_2O_7$. With similar quantities of this reagent similar products are produced by polychroming at 100°, 80°, 70° or 60° C. At 100° C. the action of $K_2Cr_2O_7$ or of Ag_2O appears to be completed in 15 minutes. In $K_2Cr_2O_7$ polychroming, H_2SO_4 can be substituted for HCl, and subsequent $BaCO_3$ neutralization removes the salts formed and prevents accidental alkali polychroming. $K_2Cr_2O_7$ polychroming produces products with narrower absorption bands than alkali polychroming.**

Having shown the importance of azure B and its eosinate in the staining of malaria parasites (Roe *et al* 1940, 1941) and correlated the staining performance for blood and plasmodia of various Romanowsky stains with their absorption spectra (Lillie *et al* 1942), it was decided to try out the acid chromate process of oxidizing methylene blue as a part of a more comprehensive study. This method has been used by MacNeal, (1906, 1925), by Scott and French, (1924), by Holmes and French (1926). These writers had been more interested in the production of azure A, azure C and methylene violet than in azure B, a dye which they considered of little value.

Starting from the results of Holmes and French, it was decided to try HCl-$K_2Cr_2O_7$ oxidations with lesser quantities of $K_2Cr_2O_7$ than they used, to see whether or not polychrome methylene blue could be produced which consisted mainly of azure B.

Hence, 5-g. portions of methylene blue (E & A)[1] were boiled 45 minutes under a reflux condenser in 300 cc. distilled water and 10 cc. concentrated (38%) HCl, adding just at the start of boiling a variable amount of 10% aqueous $K_2Cr_2O_7$ solution. At the end of the heating 1 cc. samples were removed for spectral analysis and half the sample neutralized to nitrazine paper (pH 6.8) with saturated Na_2CO_3 solution. Then the eosinate was precipitated by the addition of 23 cc. 10% eosin Y (LE-11). The eosinates were filtered out next morning using a vacuum filter, dried and dissolved in the proportion of 0.1 g. to 80 cc. C.P. methanol.

These solutions were tested on blood films containing *Plasmodium malariae*, as in the previous paper, and the results are given in the last two columns of Table 1. As noted in the earlier paper, best staining of the plasmodia occurred when polychroming of the methylene blue had proceeded to such an extent as to give an absorption maximum between 660 and 640 mμ, indicating a high proportion of azure B. In the present experiment, when the absorption maximum of the polychromed methylene blue (column 4) is taken into account it will be seen that the shift of the absorption maximum and the widening of the band, at which D was over 90%, was strictly in the order expected from the amount of $K_2Cr_2O_7$ used. Staining performance did not follow this order, however, as there were a few instances where the staining of the plasmodia was as poor as in the case of sample I-K, the unpolychromed dye—notably I-H and I-I, and to a less extent I-E and I-G, which showed the gray lilac staining of the parasite cytoplasm and the redder chromatin which would be expected of azure A stains, rather than the azure B picture which would have been expected from the spectra of the thiazin. In an effort to explain this discrepancy, absorption spectra of the thiazin eosinates were made; and comparison of these spectra with those of the freshly polychromed, still acid, methylene blue, revealed that these four samples had developed considerable shifts of their thiazin absorption spectra in the process of being converted into eosinates. This comparison further revealed that samples I-A and I-B, which unexpectedly gave better staining than sample I-C, had also undergone further polychroming in the process of preparation of the eosinate, whereas sample I-C had not.

As the differences between the first series of polychrome methylene blues and their eosinates were attributed to alkali polychroming by the Na_2CO_3 used in neutralization, it was determined to use an insoluble carbonate for neutralization. As it seemed desirable at the

[1]E & A, procured from Eimer and Amend before 1925.

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Lot No.	Graph No.	Mg. $K_2Cr_2O_7$ per g. M. B.	Thiazin			Thiazin eosinate				Staining performance†	
			λ	90% D λ band*		Eosin λ	Thiazin λ	90% D λ band of thiazin			
				Width	Median			Width	Median	Blood	Plasmodia
I-K	1, 68	0.0	664.5	18.3	662.3	516	663	18.3	661.1	±	±
I-A	2, 75	20	663.5	19.6	662.0	517	654	36.9	649.0	+++	+++
I-B	3, 76	40	663.5	19.1	662.0	516	654	35.1	653.9	+++	++
I-C	4, 77	60	662.0	21.5	660.0	517	662	24.0	660.1	+	±
I-D	5, 78	100	659.0	22.8	657.7	517	650	26.3	649.5	+++	+++
II-A	6, 158	100	658.0	23.0	656.9	516	658	23.1	656.7	+++	+++
II-B	159	120				516	657	25.7	655.7	+++	+++
II-C	160	140				516	657	26.3	654.9	+++	++
I-E	7, 79	150	655.5	24.3	654.3	517	635	40.2	637.5	+++	++
II-E	8	160	655.5	25.4	654.7	516	656	25.7	655.5	+++	++
II-F		180				516	657	26.8	656.2	+++	++
I-F	9, 80	200	654.0	26.4	651.8	517	642	37.6	640.6	+++	+++
II-G		200				516	658	25.5	656.9	+++	++
I-G	10, 81	250	651.0	31.0	647.0	517	635	35.4	633.7	+++	++
I-H	11, 82	300	649.0	31.8	646.3	517	625	33.7	624.2	+++	+
I-I	12, 83	400	642.0	44.1	636.7	517	617	30.6	616.1	+++	±

*D is the logarithm of the reciprocal of percentage transmittancy (T) and is expressed thus: D = log 1/T.
λ is the wave length at which maximum absorption occurs.
The wave-length band of 90% D λ is that band thruout which D is at or over 90% of its value at λ.
†In this column symbols signify as follows: ±, poor; +, fair; ++, good; +++, excellent.

same time to attempt to eliminate the additional salt content produced by introduction of acid and its subsequent neutralization, H_2SO_4 was substituted for HCl and $BaCO_3$ for Na_2CO_3. Calculation showed that 3.4 cc. concentrated H_2SO_4 (sp. gr. 1.835) was approximately equivalent to 10 cc. 38% HCl, and that this would be neutralized by about 11.75 g. $BaCO_3$.

Four 5-g. samples of methylene blue (E & A) were dissolved each in 300 cc. boiling distilled water containing 3.4 cc. concentrated H_2SO_4, with 7.5 cc. 10% $K_2Cr_2O_7$ added to each. These were boiled respectively 45 minutes, 1½ hours, 3 hours and 6 hours under a reflux condenser. After adding 18 g. $BaCO_3$ (a 50% excess over the theoretical amount), boiling was continued another 20 minutes. The dye solution was then filtered on a vacuum filter and the barium salts washed with several small washes of hot water, bringing the total volume to 501 cc. One-cubic-centimeter samples were taken for spectral analysis and half of each sample was precipitated with 23 cc. 10% eosin Y (LE-11) as before.

Table 2 reveals that the thiazins alone and as eosinates give essentially identical spectra and that the difference in heating time has made no appreciable difference in the product.

As the foregoing experiments had not adequately explored the azure A range in acid bichromate polychroming, nor the minimum time for completion of oxidation, and as it appeared at that time that polychroming at lower temperatures by this process did not carry the demethylation beyond azure B, an experiment was set up to fill in these deficiencies. In the determination of the time requirements spectrophotometrographic examinations were made first of the samples heated the shortest times, until no further alteration was evident; and to check on this, the sample heated the longest time was examined. While intervening samples were taken, their examination was deemed unnecessary if samples heated for longer and shorter periods were essentially identical.

Examination of the data in Table 3 indicates that at 97°C. the oxidation is virtually completed in 10 minutes and fully so in 15 minutes, while no further change occurs with 1 and 4 hours heating. One striking thing is the narrowness of the absorption bands produced by the acid oxidation process in the 620–630 mμ area as compared with those produced by the alkali processes. Substantially the same grade of change is produced by heating with 500 mg. $K_2Cr_2O_7$ per gram methylene blue for any of the following periods and temperatures: 15 minutes at 97°C.; 1 day or 1 hour at 80°C.; 8 hours at 70°C.; 1 day (or less) at 60°C. More than 500 mg. $K_2Cr_2O_7$ per gram methylene blue produces little further effect.

TABLE 2. POLYCHROME METHYLENE BLUES AND THEIR EOSINATES, VARIATION OF HEATING TIME, H_2SO_4-$K_2Cr_2O_7$ SERIES, $BaCO_3$ NEUTRALIZATION

Lot No.	Graph No.	Mg. $K_2Cr_2O_7$ per g. M. B.	Time heated	Thiazin			Thiazin eosinate			
				λ	90% D λ band*		Eosin λ	Thiazin λ	90% D λ band of thiazin	
					Width	Median			Width	Median
IV-AB	22, 92	150	45 min.	655	25.1	653.1	517	655	27.9	652.8
IV-CD	23, 93	150	90 min.	655	24.1	653.9	517	655	28.3	652.6
IV-EF	24, 94	150	3 hours	655	25.2	653.5	517	654+	28.5	653.9
IV-GH	25, 95	150	6 hours	655	23.7	653.6	517	653	27.7	651.3

*D is the logarithm of the reciprocal of percentage transmittancy (T) and is expressed thus: $D = \log 1/T$.
λ is the wave length at which maximum absorption occurs.
The wave-length band of 90% D λ is that band thruout which D is at or over 90% of its value at λ.

TABLE 3. SPECTROPHOTOMETRIC DATA ON METHYLENE BLUE SAMPLES HEATED IN 1.1% H_2SO_4 SOLUTION IN 1% CONCENTRATION WITH VARYING AMOUNTS OF $K_2Cr_2O_7$, FOR VARYING LENGTHS OF TIME AND AT 97°C., 80°C., 70°C. AND 60°C.

Graph No.	Dur. of heating	Temperature	$K_2Cr_2O_7$ per gram methylene blue	Absorpt. Maximum	90% D λ band data*	
					Width	Median
361	1 hr.	97°C.	0 mg.	662	18.8	660.5
362	"	"	50 mg.	660	19.8	659.0
363	"	"	100 mg.	658	19.0	657.7
364	"	"	150 mg.	655	23.0	654.15
365	"	"	200 mg.	650	26.4	648.1
366	"	"	300 mg.	644	32.5	641.0
367	"	"	400 mg.	634	31.5	633.2
368	"	"	500 mg.	628	29.4	627.3
369	"	"	750 mg.	625	24.3	623.9
370	"	"	1000 mg.	622	23.7	621.6
371	5 min.	"	500 mg.	632	33.6	631.5
372	10 min.	"	500 mg.	630	28.0	629.0
373	15 min.	"	500 mg.	628	28.3	626.7
374	4 hr.	"	500 mg.	629	30.3	627.7
376	1 hr.	80°C.	500 mg.	631	29.3	630.3
377	1 day	"	500 mg.	629	28.0	628.6
378	8 hr.	70°C.	500 mg.	628	28.2	627.2
379	4 days	"	500 mg.	628	33.6	627.1
380	1 day	60°C.	500 mg.	629	29.4	628.3

*D is the logarithm of the reciprocal of percentage transmittancy (T) and is expressed thus: $D = \log 1/T$.

λ is the wave length at which maximum absorption occurs.

The wave-length band of 90% D λ is that band thruout which D is at or over 90% of its value at λ.

All the foregoing acid bichromate polychroming experiments were done with a single lot of methylene blue (E & A). Hence it seemed necessary to repeat sufficient of them on various other lots of methylene blue to show the general applicability of the method.

Therefore, 1-g. lots of various methylene, ethylene and thionine blues were dissolved each in 61.7 cc. of distilled water containing 1.13 cc. concentrated H_2SO_4 (sp. gr. 1.84). One cubic centimeter was taken from each lot for spectrophotometric examination, and each lot was then divided into 5 portions of 12 cc. each, to which were added severally 1.0, 1.25, 1.5, 1.75 and 2.0 cc. of 2% aqueous $K_2Cr_2O_7$, equivalent respectively to 100, 125, 150, 175 and 200 mg. $K_2Cr_2O_7$ per g. methylene blue. These were heated simultaneously in test tubes in a bath of boiling water for 90 minutes and removed from the water bath. To each tube 0.6 g. $BaCO_3$ was added, and heating was continued for another 30 minutes. The various samples were then filtered and stored in labelled vials for spectrophotometric study. The results of these studies are shown in Table 4.

It appears that the amount of demethylation is quantitatively quite similar for the various amounts of bichromate with the modern American samples: the two lots of LA-7, the NA-8 and the uncertified lot from Hartman-Leddon Co. The sample from the H. S. Laboratory, labelled thionine blue appears to resemble more the modern American methylene blues. The old Grübler 12.13 (probably 1913) sample shows quantitatively greater demethylation, in accord with its probably lower dye content. The Eimer and Amend lot labelled ethylene blue, which term is a synonym of methylene blue, does not appear to be that dye, but resembles more the sample of thionine blue (C.I. No. 926, not thionin C.I. No. 920) obtained from the Commission on Standardization of Biological Stains in its absorption maximum, but its demethylation is apparently limited rather than progressive as with thionine blue. (Thionine blue is described as ethyl trimethyl thionin, and the first dealkylation product would appear to be an ethyl dimethyl thionin rather than trimethyl thionin which is azure B.)

In the course of this study it seemed of interest to determine what products were produced in the Wilson[2] method of polychroming with Ag_2O at boiling temperatures. Since the Wilson procedure calls for removal of one-third after 30 minutes heating, another third after 60 minutes and the balance after 90 minutes, these three time intervals were used on separate portions. Ag_2O was used with 0.5% $NaHCO_3$, as in the Wilson procedure, and also without the carbonate. Each combination was run in duplicate at different times, making a total of 12 lots. The technic was as follows: To 1 g. $AgNO_3$ in 8 cc. distilled water, add 0.2 g. NaOH in 2 cc. distilled water; let settle 20

[2]The earliest reference to this method which the author has been able to locate is in Stitt's Practical Bacteriology, Blood Work and Parasitology, *5th Ed.* (Philadelphia, 1918), pp. 239–40.

TABLE 4. SPECTROPHOTOMETRIC DATA ON VARIOUS LOTS OF METHYLENE BLUE, ETHYLENE BLUE AND THIONIN BLUE, POLYCHROMED 90 MINUTES AT 97°C. BY THE H_2SO_4-$K_2Cr_2O_7$ PROCESS WITH $BaCO_3$ NEUTRALIZATION

	$K_2Cr_2O_7$ per g. dye in mg.	Methylene blue samples†					Ethylene blue†	Thionine blue†	
		Lot VI-A H. L. Co. LA-7	Lot VII-C H. L. Co. LA-7	Lot VI-B Grübler 12.13	Lot VI-D N. A. Co. NA-8	Lot VI-E H. L. Co.	Lot VI-C E. & A.	Lot VII-A Mfg. unknown	Lot VII-B H. S. Labs.
Absorption maximum (λ)	0	665.0	664.0	664.0	665.0	663.5	668.0	668.0	664.0
	100	665.0	659.0	659.0	660.0	659.0	662.0	663.0	659.0
	125	658.0	657.0	656.0	656.0	658.0	663.0	663.0	657.0
	150	656.0	655.5	653.0	655.5	656.0	662.0	661.0	656.0
	175	654.0	656.0	652.0	653.0	655.0	660.0	659.5	655.0
	200	653.0	654.0	650.0	653.0	653.0	660.0	657.0	652.0
90% D λ band width*	0	18.0	18.4	19.2	18.1	18.9	16.8	13.1	20.4
	100	19.4	24.1	24.5	22.4	22.8	21.4	19.6	22.8
	125	21.6	24.1	24.4	24.7	23.5	21.1	21.6	25.0
	150	24.3	24.3	26.5	25.5	24.7	22.5	22.9	25.4
	175	24.3	26.7	25.8	24.4	25.4	23.5	22.9	24.0
	200	27.4	24.7	26.4	25.8	26.0	22.0	26.9	25.9

Median of 90% D λ band	0	663.3	662.5	663.1	662.9	662.8	666.8	668.7	663.1
	100	663.2	657.5	656.3	658.1	658.1	659.0	662.3	657.5
	125	657.6	656.2	654.9	654.9	656.8	661.6	661.6	655.8
	150	655.4	655.1	651.8	653.8	655.0	660.0	659.2	653.9
	175	652.5	653.3	650.0	652.5	653.4	659.6	658.8	652.6
	200	652.2	651.9	648.2	651.3	650.3	659.3	656.0	650.1

TABLE 5. EFFECT OF OXIDATION WITH $KMnO_4$ AT 98°C. ON THE ABSORPTION SPECTRUM OF METHYLENE BLUE IN ACID SOLUTION

Graph No.	$KMnO_4$ per g. M. B.	Absorption Maximum	90% D λ band*		Dilution and D λ actual	Dilution to give D λ = 1.000
			Width	Median		
384	250 mg.	660	20.0 mγ	659.5	2 γ/cc. .6861	2.9 γ/cc.
385	500 mg.	657	23.8	656.8	2.6 γ/cc. .5638	4.6 γ/cc.
386	750 mg.	657	26.6	656.3	3 γ/cc. .5560	5.4 γ/cc.
387	1000 mg.	654	29.2	650.3	4.4 γ/cc. .5376	8.2 γ/cc.
390	500+1000 mg.	648	34.6	643.8	28.5 γ/cc. .5560	51.3 γ/cc.
391	500+1000+500 mg.	614	32.9	614.5	100 γ/cc. .7012	142.6 γ/cc.

*D is the logarithm of the reciprocal of percentage transmittancy (T) and is expressed thus: D = log 1/T.
λ is the wave length at which maximum absorption occurs.
The wave-length band of 90% D λ is that band thruout which D is at or over 90% of its value at λ.

†The abbreviations indicating manufacturers of these samples are as follows: H. L., Hartman-Leddon of Philadelphia; N. A., National Aniline; E. & A., Eimer and Amend; H. S. Labs., the H. S. Laboratories of Philadelphia. The sample of thionine blue of unknown manufacture was obtained from the laboratory of the Stain Commission at Washington, D. C. in 1940.

TABLE 6. SPECTROPHOTOMETRIC DATA ON EOSINATES OF METHYLENE BLUE POLYCHROMED WITH SILVER OXIDE

Graph No.	Lot No.	$NaHCO_3$ g.	$AgNO_3$ g.	M. B. g.	Temp. °C.	Time heated min.	Absorption maxima		90% D λ band of thiazin*	
							Eosin	Thiazin	Width	Median
191	IX-G	0.5	1.0	1.0	100	30	517	618	34.7	618.8
193	IX-H	0.5	1.0	1.0	100	30	516	616	37.6	619.5
195	IX-I	0.5	1.0	1.0	100	60	517	614	31.0	612.9
198	IX-J	0.5	1.0	1.0	100	60	517	614	31.0	614.3
201	IX-K	0.5	1.0	1.0	100	90	517	614	30.6	613.4
205	IX-L	0.5	1.0	1.0	100	90	517	615	32.9	616.6
181	IX-A	0	1.0	1.0	100	30	517	620	49.8–	628.4
182	IX-B	0	1.0	1.0	100	30	517	624	45.0	632.1
184	IX-C	0	1.0	1.0	100	60	516	621	44.4	628.7
185	IX-D	0	1.0	1.0	100	60	516	622	47.6	627.2
187	IX-E	0	1.0	1.0	100	90	517	620	37.6	621.8
189	IX-F	0	1.0	1.0	100	90	516	622	44.3	626.2
206	IX-M	0	1.0	1.0	100	15	516	622	43.0	623.7
207	IX-N	0	1.0	1.0	80	15	516	621	40.2	622.0
216	X-A	0	1.0	1.0	97	60	516	622	44.0	626.9

224	X-I	0	0.5	0.5	97	60	517	622	37.9	622.3
217	X-B	0	0.8	1.0	97	60	516	628	53.5–	635.5
225	X-K	0	0.4	0.5	97	60	517	621	38.8	623.4
218	X-C	0	0.6	1.0	97	60	517	650	54.9+	638.9
226	X-L	0	0.3	0.5	97	60	517	623	46.2	627.4
219	X-D	0	0.5	1.0	97	60	517	652	53.8	639.0
227	X-M	0	0.25	0.5	97	60	517	634	50.6	631.1
220	X-E	0	0.4	1.0	97	60	516	655	36.0	650.6
228	X-N	0	0.2	0.5	97	60	516	632	56.2	633.6
221	X-F	0	0.3	1.0	97	60	516	649	53.0	634.9
229	X-O	0	0.15	0.5	97	60	516	629	56.9	634.2
222	X-G	0	0.2	1.0	97	60	516	656	32.7+	651.8
230	X-P	0	0.1	0.5	97	60	516	655	36.6–	650.2
223	X-H	0	0.1	1.0	97	60	516	660	25.6	655.9
231	X-R	0	0.05	0.5	97	60	516	656	35.4	651.2

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The wave-length band of 90% D λ is that band thruout which D is at or over 90% of its value at λ.

minutes; pipette off and discard supernatant fluid; wash 4 times by adding 100 cc. distilled water each time, letting settle 20 minutes each time as before, and pipetting off supernatant fluid. To the moist Ag_2O add 100 cc. 1% solution of methylene blue in distilled water or in 0.5% $NaHCO_3$. Boil 30 minutes, adding small quantities of distilled water frequently to maintain volume.

Spectrophotometric data are presented in Table 6.

The results of the polychroming for 30, 60 and 90 minutes without $NaHCO_3$ are quite similar in the six lots, and also in two additional lots heated 15 minutes at 100° and at 80°C., giving thiazin absorption maxima between 620 and 624 mμ, and 90% Dλ bands about 37 to 50 mμ in width with medians between 622 and 632 mμ. With both $NaHCO_3$ and Ag_2O the alteration was consistently greater, and quite similar in the six lots, giving thiazin absorption maxima between 614 and 618 mμ and 90% Dλ bands 30 to 37 mμ in width with medians between 612 and 620 mμ.

In view of this information, the Wilson procedure of combining products heated 30, 60, and 90 minutes seems superfluous, as the maximal effect of Ag_2O (without $NaHCO_3$) seems to be evident after only 15 minutes even at 80°C.

At this point the question came up whether Ag_2O might not act quantitatively as an oxidizing agent, in the same manner as does $K_2Cr_2O_7$ in acid solution. To determine this question a series of lots of polychrome methylene blue was made by heating simultaneously, for 1 hour in an Arnold steam sterilizer at about 97°C., 16 lots of 1% methylene blue with the Ag_2O prepared as before from variable amounts of $AgNO_3$. The data presented in the second half of Table 5 show more irregularity than similar series with $K_2Cr_2O_7$, but it is plain that the larger amounts of Ag_2O cause the larger shift in the absorption maximum, and the lesser, smaller displacements. Irregularities may be at least partly explained by losses of Ag_2O in the process of its preparation and washing.

At this point some further exploration of other oxidizing agents as to their action on methylene blue was deemed advisable. As $KMnO_4$ has been used[3], this substance was selected for trial. A preliminary lot was made by boiling 100 mg. methylene blue, 33 mg. $KMnO_4$ and 0.02 cc. concentrated HCl in 20 cc. water for 5 minutes. This gave an absorption maximum at 655–660 and a 90% Dλ band width of 21.5 mμ with median at 657.9.

As these results appeared encouraging, a series of 10 lots of 100 mg.

[3]I recall the statement in an old French hematology text, which I have been unable to find since.

methylene blue each in 10 cc. 0.1% H_2SO_4 were heated 1 hour in a bath of boiling water with the following amounts of $KMnO_4$: 0, 5, 10, 15, 20, 30, 40, 50, 75 and 100 mg. As only a slight shift in absorption spectra occurred even with the largest amount of $KMnO_4$, the tubes were reheated for 2 hours more which produced no further change, the 100 mg. $KMnO_4$ lot still showing an absorption maximum at 658 mμ (659 at 1 hour), and a 90% Dλ band 22 mμ wide (22.5 at 1 hour) with median at 657.6 (657.5 at 1 hour). As the use of 0.1% H_2SO_4 had been due to an error in calculation, it was decided to repeat this experiment using 1.1% H_2SO_4 as the equivalent of the 3.4 cc. to 300 cc. which was used in the previous $K_2Cr_2O_7$ experiments. Satisfactory samples were obtained using 250, 500, 750 and 1,000 mg. $KMnO_4$ per gram methylene blue, but when 2.5 g. per gram of the dye was used, a brown granular precipitate was promptly produced which failed to redissolve with several hours heating. Addition of another gram of $KMnO_4$ to the lot polychromed with 500 mg. per gram dye left a blue dye after evolution of gas had ceased; therefore, after an hour at 98°C., a sample was taken for absorption spectrum, and after adding 500 mg. $KMnO_4$, was heated for another hour. Then another sample was taken and a further heating with an additional 500 mg. $KMnO_4$ followed, leaving much brown precipitate and only a faint color in the fluid. (See Table 5.)

The dilution data indicate that in addition to demethylation of part of the dye, much of it is entirely destroyed. This is confirmed by the fact that immediate or fractional addition of 2.5 g. $KMnO_4$ to 1 g. dye leaves a black granular residue in a water white solution.

HgO appeared to be inert when used with methylene blue dissolved in 1.1% H_2SO_4 or in distilled water using as much as 1.2 g. HgO per g. methylene blue in each case. Were the oxygen in this compound available, the quantity used should have been sufficient to oxidize 2 methyl groups and to form azure A. Absorption maxima remained at 662 as in the untreated methylene blue. $NaIO_3$, (480 mg. per gram methylene blue, enough theoretically to oxidize 2 methyl groups) was also inert.

Summary

Various lots of methylene blue, when heated with similar quantities of $K_2Cr_2O_7$ in acid solution yield closely similar products, and larger amounts produce quantitatively greater changes.

In polychroming with $K_2Cr_2O_7$ in acid solution the process appears to be completed in 15 minutes at 100°C. and no further change occurs with prolonged further heating. The same end product is formed with the same amount of bichromate whether the heating is at 100°,

80°, 70° or 60°C. More than 500 mg. $K_2Cr_2O_7$ per g. methylene blue produces little further effect at temperatures up to 100°C. The products generally present much narrower spectral absorption bands than do the products of alkali polychroming with similar absorption maxima. The use of H_2SO_4 in place of HCl, and subsequent $BaCO_3$ neutralization gives quite satisfactory results, avoids the possibility of further polychroming which may occur if Na_2CO_3 is used, and removes the mineral salts effectively from the final solution.

Ag_2O, when used alone, is similar to $K_2Cr_2O_7$ in its action, in that at 100°C. its action is completed in about 15 minutes and prolonged further heating gives no further alteration in the absorption spectrum, and further in that the amount of alteration produced is roughly proportional to the amount of Ag_2O used.

In 0.1% H_2SO_4 solution $KMnO_4$ produces little change in methylene blue, but in 1.1% H_2SO_4 polychroming occurs which is roughly proportional to the amount of $KMnO_4$ used. However, at the same time much of the dye is totally destroyed, prolonged oxidation with an excess resulting in a colorless solution and a black granular residue.

HgO and $NaIO_3$ produce no polychroming of methylene blue at 100°C. either in neutral or in acid solution.

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THE MARCHI METHOD

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ABSTRACT.—**An investigation has been made of the poor penetration obtained by the Marchi method. Use of surface tension depressants or perfusion technics showed no marked improvement. Optimum results cannot be achieved with slices of tissue above 3 mm. in thickness. Another cause of faulty staining is the failure to maintain an adequate strength of OsO_4 in the Marchi mixture. The concentration of this chemical should never be allowed to drop below 0.25%. A chemical method for determining the amount of OsO_4 present in the staining mixture and based upon the Alvarez test is described. This method can also be utilized to reclaim partially exhausted staining solutions. Remarks are included upon the KIO_3 method of Busch.**

Among histologic methods employed upon the central neural system, the method of Marchi has remained peculiarly refractory to improvement. Altho some fundamental information has been acquired by Swank and Davenport[1] in regard to cat and rabbit material, and certain modifications of the method have been suggested for particular purposes, the essential difficulty of the technic, notably poor penetration, has not been circumvented. In so far as brain material itself is concerned, especially primate specimens, we still find the method described ten years ago in this journal[2] to be the most useful for general laboratory purposes, but many critical sections have had to be discarded because of poor penetration.

Whereas it is generally admitted that good penetration can usually be obtained upon slices of tissue 3 mm. or less, it is not always advisable to cut large brains into such thin slices because of subsequent warping while in the osmium solution. In an effort to increase the penetration of the osmium-dichromate mixture we have consequently conducted a series of experiments with surface tension depressants. Of these the most useful is "Tergitol 08" (Carbide and Carbon Chemicals Corporation, 30 East 42nd Street, New York,

[1]Swank, R. L., and Davenport, H. A. 1934–5. Marchi's staining method: I. Studies of some of the underlying mechanisms involved; II. Fixation; III. Artefacts and effects of perfusion. Stain Techn., 9, 11–9, 129–35; 10, 45–52.

[2]Mettler, Fred A. 1932. The Marchi method for demonstrating degenerated fiber connections within the central nervous system. Stain Techn., 7, 95–106.

N. Y.). This may be added directly to the 3% dichromate solution which is used for both the preliminary treatment and in making up the osmium mixture. It should be used in the proportion of one part of "Tergitol" to 150 parts of 3% dichromate. While the effect of a surface tension depressant seems to be beneficial, the improvement in penetration is not great enough to warrant recommending it as a modification of the routine procedure.

Among the modifications suggested by Swank and Davenport we have had the best penetration by the use of the following preliminary procedure. The animals (monkeys) were killed by an overdose of "Nembutal" and were perfused with a mixture of Mueller's fluid and $CuSO_4$. The brains were fixed in formalin. Altho a slightly better degree of penetration was encountered after such a procedure than after the routine technic, the difference was not marked and the tissue had a tendency to overstain. In cases in which we perfused the brain with $MgSO_4$ after death from "Nembutal" and then fixed in formalin followed by the chlorate mixture, the infiltration was very poor and the staining of the penetrated areas was suppressed. Brains from monkeys killed by exsanguination and fixed in formalin, followed by staining in a mixture of $KClO_3$, acetic acid, formalin and OsO_4 exhibited a tendency toward overstaining and underpenetration.

Swank has pointed out that good results can be obtained by transferring unperfused brains, fixed in formalin, directly to the Marchi mixture (equal parts of 3% $K_2Cr_2O_7$ and 1% OsO_4) without previous washing. Such a technic is applicable to primate material and is especially useful on monkeys that have not run the full degeneration period of two weeks. Altho we regard washing after formalin fixation and treatment with plain $K_2Cr_2O_7$ as an optional procedure, the results obtained are usually more uniform if these steps are included. Washing out of the formalin certainly reduces the tendency toward surface blackening (the brain should never be allowed to fix in formalin alone for more than 24 hours) and a stay of two weeks in $K_2Cr_2O_7$ improves the quality of the background of the finished sections. The use of plain $K_2Cr_2O_7$ can, however, be a source of trouble in that if the tissue is left in this solution too long the staining reaction is suppressed. Further, if the $K_2Cr_2O_7$ is too concentrated, the blocks of tissue become hard and recalcitrant to embedding. Very thoro washing after treatment with the osmium mixture is a necessity if good pyroxylin infiltration is to be attained.

Testing the strength of the Marchi mixture. The tendency to conserve a reagent as expensive as osmium is no doubt responsible for many cases of insufficient staining. One should always use at least

twenty times as much of the osmium-dichromate mixture as the volume of tissue to be stained, and results are likely to be more satisfactory if fifty times the tissue volume is employed. When one is working with primate brains and the cost of osmium involved is considerable, one is loathe to dispose of the partially exhausted fluid after the brain is removed from it. So far as we are aware, no one has ever suggested a method by which the unused osmium may be reclaimed. Some workers have long used weakened mixtures in place of straight 3% $K_2Cr_2O_7$ in the preliminary treatment, but this does not represent any saving in the cost of the total technic.

Not only would it be desirable to know how much osmium remains in such a mixture, but it must be admitted that there is no satisfactory way of knowing if the osmium-dichromate mixture becomes exhausted during the two-week period while the tissue is supposed to be staining. Few people can tell much about the concentration of the Marchi mixture by smelling it. A method for the detection of osmium has been described by Pinerua Alvarez[3] and can be modified to serve this purpose. The modified procedure is as follows: To 1 cc. of the osmium-dichromate mixture of unknown strength add 250 mg. of barium acetate powder, centrifuge for 10 minutes and separate the supernatant fluid from the precipitate. In order to wash out the OsO_4 which may have been adsorbed by the resulting $BaCrO_4$ precipitate, add 1 cc. of acetic barium acetate solution (saturated solution of barium acetate diluted with water 1:5 and further acidulated by the addition of a few drops of glacial acetic acid to each 100 cc. of the solution) to the precipitate, mix thoroly and centrifuge for 10 minutes. Add the supernatant fluid to that previously obtained and add 4 drops of the test solution (2 parts by volume of 20% KI plus one part of concentrated HCl). A deep emerald green color indicates the presence of osmium. While the test solution can be added directly to a sample of the Marchi mixture, it is preferable to remove the $K_2Cr_2O_7$ as indicated above[4] since it obscures the color developed in the actual test. The intensity of this color is roughly proportional to the amount of osmium present and, in laboratory practice, it will be found desirable to run three samples, notably the osmium-dichromate of unknown strength and two samples of known strength (one containing equal parts of 3% $K_2Cr_2O_7$ and 1% aqueous OsO_4, and the other containing equal parts of 3% $K_2Cr_2O_7$ and 0.5% OsO_4). In this way the unknown can be compared against standards representing 0.5% and 0.25% of OsO_4

[3]Alvarez, P. 1905. Sur un nouveau composé osmieux et une réaction de l'osmium. Compt. Rend. Acad. Sci., 140, 1254–6.

[4]We are indebted to Dr. H. B. Waelsch for assistance in this matter.

respectively. For effective staining the osmium concentration should not be allowed to drop below 0.25%.

The standards should be run at the same time as the unknown since the color of the reaction is unstable and comparison would otherwise be impossible. It is necessary to use standards containing $K_2Cr_2O_7$ since a certain amount of osmium is lost during the extraction of this substance from the unknown. If the standards also contain $K_2Cr_2O_7$ and are subjected to the extraction process, the loss is identical in each case and may be ignored in the final comparison.

Reclaiming partially exhausted staining mixtures. When one has finished staining in a particular osmium mixture, the residual osmium may be conserved in the following manner: Centrifuge to eliminate debris and compare colorimetrically the partially exhausted Marchi mixture with a 1.5% solution of $K_2Cr_2O_7$. If the percentage of $K_2Cr_2O_7$ has previously been decreased by the addition of significant amounts of 1% OsO_4, it will now be necessary to adjust this by the addition of sufficient 3% aqueous $K_2Cr_2O_7$. Test the osmium concentration as previously indicated. If the concentration of osmium is below 0.25%, it will probably not be worth while to attempt to adjust the solution to standard strength unless the volume is very large. The strength of osmium may now be brought up to the required amount by the addition of solid OsO_4 or a solution of this. If much 1% solution is added, the $K_2Cr_2O_7$ concentration has to be adjusted; so, in practice, it is better to work with amounts of partially exhausted solution aggregating a liter or more. In this case the amount of solid necessary to reënforce the solution can be computed without much difficulty, and it is consequently unnecessary to disturb the dichromate concentration. If frequently reused, the $K_2Cr_2O_7$ of any given Marchi solution will become reduced and ineffective. This will be quickly noticed since the solution appears greenish. Altho a slightly green mixture is still active and no harm is done if the change occurs while the tissue is being stained, such a solution should not be used again.

Embedding of Marchi material. Since the conventional paraffin solvents have a generally deleterious effect upon Marchi material, the pyroxylin technic is usually employed. Impregnation with a celloidin-like substance is frequently difficult to carry out on dichromate material. It is probably always advisable to remove pia by slicing away the periphery of a block wherever possible. If the block is large and this is impossible, it is better to use the method of Busch[5]

[5]Busch, C. K. 1898. Über eine Färbungsmethode secundärer Degenerationen des Nervensystems mit Osmiumsäure. Neurol. Zentbl., 17, 476.

since iodate material is less resistant to infiltration than dichromate material. If one insists upon the dichromate technic, good sections can always be obtained by taking ones time with the process of embedding. We have left Marchi material in 2% celloidin as long as a year and still obtained good sections. It is unnecessary to be in a hurry during embedding and impatience may be disastrous.

In spite of theoretic objection, celloidin blocks of Marchi stained material may be stored in glycerin and alcohol for a short time without much impairment in the contrast of the stain. We do not recommend leaving them more than a month. If it is desired to keep the tissue in blocks longer than this, terpineol is preferable. Terpineol or butyl alcohol may be used after 90% alcohol to clear sections which are difficult to carry thru absolute alcohol. All clearing agents, in time, affect the stain to some extent. In addition to those mentioned as useful in our 1932 article, we may now add oil of origanum which is better than carbol-xylene but not so good as $CHCl_3$. If one employs a quick drying mount, such as damar instead of balsam, the solvent action of the clearing agent will be found to be noticeably reduced.

Bleaching. The attempt to bleach overstained sections is generally a useless procedure. The photographic reducers, whether proportional or cutting, do not have any appreciable effect upon such sections, but H_2SO_3 or K_2SO_3 may be tried if one happens to have a few critical sections which it is desirable to salvage.

H_2O_2 is of no value in bleaching Marchi sections, but is a very useful reagent to have on hand. Occasionally a stock bottle of 1% aqueous solution of OsO_4 will become darkened. Under ordinary circumstances such a solution would be discarded, but this is unnecessary since the addition of a small amount of H_2O_2 not infrequently restores the water-like clarity of the solution. Of course, organic material and metals should be kept out of stock bottles.

Degeneration. Occasionally, when a suitable animal dies before the appointed time set for degeneration, one is tempted to stain the material in the hope that a usable result will be obtained. This practice is to be deprecated as a waste of time and osmium. What one gets, under such circumstances, is a little local staining about the immediate lesion and nothing more. Cut myelinated fibers seem to become osmium-susceptible rather suddenly. In mammals this does not appear to occur earlier than the tenth day. After three weeks good results are secured with primate material and acceptable sections can be obtained as long as five weeks after lesion. (We do not consider the possibility of retrograde degeneration to be a serious

enough objection to discard material which has run for this length of time; tho the record of the case should always clearly state the time period involved). If one is forced to stain before the lapse of the optimal period, Swank's recommendation of eliminating preparatory mordanting in 3% $K_2Cr_2O_7$ should be adopted, but negative results should not be reported on such material.

Potassium iodate technic (method of Busch). The use of KIO_3, in place of $K_2Cr_2O_7$, possesses the advantage of more complete pyroxylin infiltration and consequently greater speed. It has the disadvantage of giving an exceptionally pale background (which may, however, be counterstained).

We have found the following to be the best procedure: (1) fix in an aqueous solution of 10% formalin and 1.5% KIO_3 for about 24 hours; (2) rinse briefly, cut into slices not above 4 mm. thick and place directly in an aqueous solution consisting of 2 parts of 1.5% KIO_3[6] and one part of 1% aqueous OsO_4 solution; (3) after infiltration with the osmium-iodate mixture for about two weeks, wash thoroly in running water until all traces of the KIO_3 have been removed, dehydrate in short steps, slowly and completely, and run up in pyroxylin, remembering that most of the actual infiltration occurs in the weakest concentrations. Counterstain, if desired, with carbolfuchsin or safranin.

When the method has been properly applied, the degree of background protection obtained by the iodate technic exceeds that resulting from the use of dichromate. If the KIO_3 has decomposed, the osmium will stain the interior of the block indiscriminately and only the periphery will show adequate protection.

[6]Caution: do not use $KH(IO_3)_2$ and do not mix the KIO_3 until ready to use since it deteriorates and proportional protection of normal tissue will not be obtained. Further, do not wash between steps 1 and 2 since this is likely to cause considerable softening and consequent distortion. A preliminary iodate solution placed between simple formalin fixation and the Busch mixture should not be used but, if the iodate was omitted from the fixer, go, after a single water rinse, directly to the Busch mixture from formalin.

A FLAGELLA STAINING TECHNIC FOR SOIL BACTERIA

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ABSTRACT.—**In an attempt to stain the flagella of soil bacteria, many of which have flagella so fine that they are hard to stain by most methods, a technic was developed which combines the best points of Hofer and Wilson's method with that of Bailey as developed by O'Toole. Satisfactory preparations have been obtained for organisms of the genera *Pseudomonas*, *Phytomonas*, *Alcaligenes*, *Escherichia*, *Azotobacter*, and *Bacillus*. This technic, therefore, is recommended as a rapid and constant method for routine flagella staining of all motile aerobic organisms; it combines Hofer and Wilson's method of cleaning the slides with O'Toole's technic of spreading the smears and with a modification of Bailey's mordant.**

This technic was developed in an attempt to stain soil bacteria, many of which have flagella so fine that they are hard to stain by the methods previously described. Altho much has been written about flagella staining, very little work has been done with soil bacteria. Many of the common soil and water bacteria have been described as motile but with no flagella reported. With other organisms, flagella have been observed, but the type of flagellation has been reported differently by various investigators, probably because the cells and the flagella are so small that the staining is not satisfactory. These difficulties indicated that further work along this line was necessary. One step in this direction was the modification of Gray's method (1926) by Hofer and Wilson (1938) for staining slime-forming organisms. This was further modified by Conn and Wolfe (1938) for use with other types of bacteria. This technic has several disadvantages. First, the method employed in spreading the smears results in so few bacteria per field that a great deal of time is required for the examination of one slide. Furthermore, the method has proved to be extremely variable, possibly due to the use of saturated solutions in the mordant. In addition, the background is often heavily covered with precipitate.

In searching for a better method, that of Bailey (1929), as modified by O'Toole (1942) for use with anaerobes, was tried. Altho

[1]Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 512.

this technic had several advantages over the others, it was not found to be suitable for bacteria having fine flagella.

In the present study, the test organism was an unidentified pseudomonad which was actively motile in hanging drop but (probably because of unusually fine flagella) was difficult to stain by all methods tried. In an attempt to stain this organism, the above methods were modified and combined in various ways. Different combinations of the chemicals were tried in the mordant. Those which proved most satisfactory were tried in different concentrations, and solutions of a definite strength were substituted for the saturated solutions (which vary with temperature changes). Finally, the most satisfactory length of time for each step was determined.

Recommended Procedure

As a result of this work the following method is recommended.

Cleaning of slides: Use new slides if available. Wash in dichromate cleaning solution; rinse in distilled water; transfer to 95% alcohol. Upon removal from the alcohol, heat the slide by passing back and forth thru a flame until a yellow color appears in the flame. Gradually lift the slide above the hottest part of the flame and then place, flamed side up, on a metal plate, heated just hot enough to produce steam from water. Allow the plate to cool gradually in order to re-anneal the slide and thus prevent breakage. Some experience is required to judge the amount of heating necessary. This part of the technic is a modification of that described by Hofer and Wilson.

Preparation of cultures: Use young actively growing cultures on agar slants (the exact age depends upon the species studied; approximately 18 hours' incubation is satisfactory for most organisms). Before proceeding, check the culture for motility by the hanging drop method. Wash off the growth by gentle agitation with 2–3 ml. of sterile distilled water. Transfer the suspension to a sterile test tube and incubate at optimum temperature for 10 minutes (30 minutes for those producing slime). At this point, again observe the suspension for motility. Transfer a small drop of the culture from the top of the suspension by means of a Pasteur pipette to one end of the previously cleaned and cooled slide. Gently tilt the slide and allow the drop to run slowly to the other end. When properly spread, there is room for three such streaks on a slide. Place the slide in a tilted position and allow to dry in the air. This method of spreading was first described by O'Toole, and gives better results than the method of Conn and Wolfe. The reason for this is that O'Toole's technic gives a longer margin per unit area than the other method,

and since it is a well-known fact that motile organisms tend to migrate toward the edge of the smear, it is comparatively easy to find a good field.

Mordanting: The method used in mordanting is a modification of the Bailey-O'Toole procedure, which requires two solutions that should be prepared fresh each time.

Solution I

Tannic acid, 10% aqueous	18 ml.
Ferric chloride, 6% aqueous	6 ml.

Solution II

Solution I	3.5 ml.
Basic fuchsin, 5% alcoholic	0.5 ml.
HCl, concentrated	0.5 ml.
Formalin	2.0 ml.

In this formula, the most important modification is the substitution of a 5% alcoholic solution of basic fuchsin for Ziehl-Neelsen's carbol fuchsin as recommended by Bailey. The mordant is applied as follows: Pour Solution I onto the slide thru a filter and allow it to remain for 3.5 minutes. Pour off this solution, and without washing, add Solution II in the same way. After 7 minutes, wash the slide with distilled water. These solutions may be refiltered and used again as many times as desired in any one day, but must not be kept overnight.

Staining: Before the slide dries, cover with Ziehl-Neelsen's carbol fuchsin, and allow it to stand for one minute on a hot plate heated just enough for steam to be barely given off. (This is essentially as recommended by Bailey). Wash and dry the slide in the air.

The time required for the first step of the mordant and for the staining should be carefully controlled because a slight variation either way may have a marked effect on the amount of precipitate and the intensity of staining.

Organisms used: In addition to the pseudomonad used as the test organism, this method was tried with *Alcaligenes fecalis*, *Phytomonas tumefaciens*, *Phytomonas savastanoi*, *Pseudomonas fluorescens*, *Escherichia coli*, *Azotobacter vinelandii*, and *Bacillus subtilis*[2]. The above organisms were chosen as representatives of the major groups of motile aerobic bacteria.

[2]The organisms listed were obtained from the following sources: *Alcaligenes fecalis*, Dr. Carl Nyberg, Helsinki, Finland; *Phytomonas tumefaciens*, Dr. Stapp, Berlin, Germany; *Phytomonas savastanoi*, Clayton Smith, Riverside, Calif.; *Pseudomonas fluorescens*, stock culture, N. Y. Agricultural Experiment Station; *Escherichia coli*, American Type Culture Collection; *Azotobacter vinelandii*, N. R. Smith, Washington, D. C.; *Bacillus subtilis*, E. H. Wheeler, Hobart College, Geneva, N. Y.

Results

The test organism, when stained by this method, proved to have a single, fine, very long, polar flagellum; the authors, therefore, believe that it belongs to the genus *Pseudomonas*. Photomicrographs of this organism are shown in Fig. 1. *Pseudomonas fluorescens*

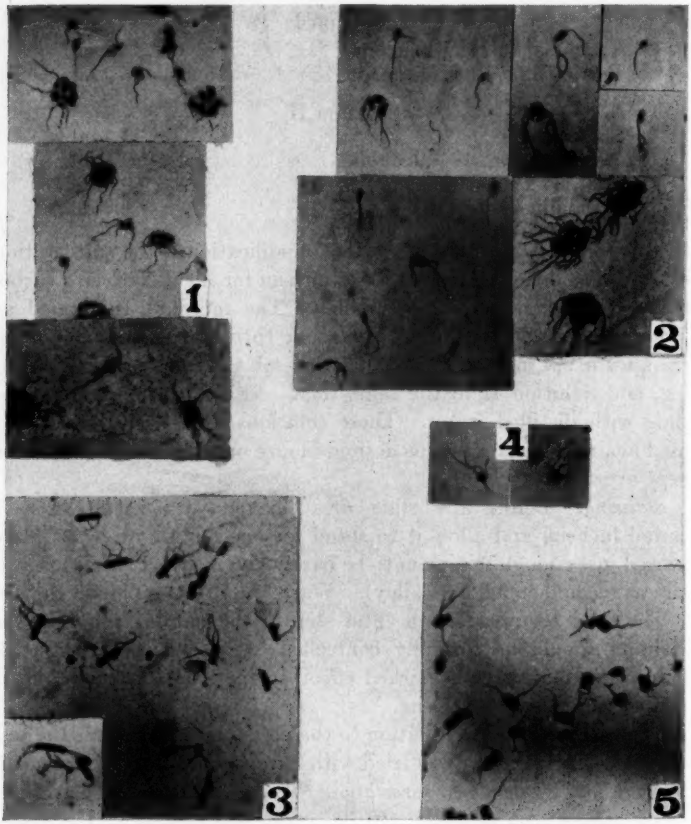

Fig. 1. The test organism, an unidentified species of *Pseudomonas*. ×900.
Fig. 2. *Pseudomonas fluorescens*. ×900.
Fig. 3. *Alcaligenes fecalis*. ×900.
Fig. 4. *Escherichia coli*. ×900.
Fig. 5. *Azotobacter vinelandii*. ×900.

was easier to stain, and many fine preparations were obtained (Fig. 2). Excellent results were obtained with *Alcaligenes fecalis*,

which proved to be a good example of an organism having "degenerate peritrichous" flagellation (Fig. 3). The flagella on the two species of *Phytomonas* showed up very nicely, altho both the organisms and the flagella were so small that no good pictures of them were secured. This method, like most other flagella staining technics, worked well with *Escherichia coli* (Fig. 4). *Azotobacter vinelandii* showed large cells with "degenerate peritrichous" flagellation which was fairly easy to demonstrate by staining (Fig. 5). This organism has previously been described by Lipman (1903) as having a single polar flagellum. The staining of *Bacillus subtilis* was not difficult; the only problem encountered was that of obtaining the culture at the right age.

Conclusions

This staining technic proved to give consistently good results, and showed the flagella distinctly outlined against a clear background. Even those species of *Phytomonas* and *Pseudomonas* that have flagella which are not readily observed by other methods are easily stained by this technic. Since satisfactory preparations were obtained for such a wide variety of organisms, it is reasonable to assume that the method can be used for any motile aerobic species.

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A RAPID SILVER-ON-THE-SLIDE METHOD FOR NERVOUS TISSUE[1]

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ABSTRACT.—**A progressive silver staining method is described, which permits microscopic examination of the sections during the staining process. After formaldehyde fixation, dehydration and embedding in paraffin or celloidin, fine fibers and synaptic endings may be demonstrated. After formaldehyde fixation and mordanting in 3% $K_2Cr_2O_7$, myelinated fibers and mitochondria are specifically stained.**

The unique feature of this method is, that the silver solution (0.5% protargol) is mixed with the reducing solution: 1.6% Rochelle salts, containing traces of Ag NO_3, $MgSO_4$, and K_2S (U.S.P.). The sections are placed directly into this mixture, which is then warmed to 45–55° C. Sections are removed when progressive staining is completed, washed in water, dehydrated and mounted.

In the fiber stain, nerve fibers and synaptic endings are dark brown or black, and nuclear chromatin is deep brown, against a pale yellow background. When the myelin sheath procedure is followed, the fiber bundles are deep brown, and the intensity of the staining remains the same for specific tracts, aiding in their identification.

This method offers the advantages of speed, simplicity, and progressive staining in the demonstration of fine fibers and synaptic endings in the nervous system.

With appropriate modification in fixation and dehydration, it may also be used as a specific stain for myelinated fibers. The theoretical considerations underlying the use of this, and other silver methods, have already been discussed. (Silver, 1942).

The silver solution used in this method, protargol (silver albumose), is not new in histological technic. It was first used by Regaud and Dubreuil (1903), rediscovered by Bartelmez and Hoerr (1933), and since used by Bodian (1936), Davenport *et al.* (1938, 1939), and others.

The reducing solution used in this method is new to histological technic. It is derived from an old formula for silvering glass, for

[1]This work was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

which I am indebted to my friend, Victor Shanok, who initiated me into the fine art of chemical reduction of silver. It differs from other reducing solutions used in histological silver methods in composition, and especially in its method of use. It is mixed directly with the silver solution, the slides are immersed in the warmed mixture, staining is progressive, and in 2–3 hours the stain is completed.

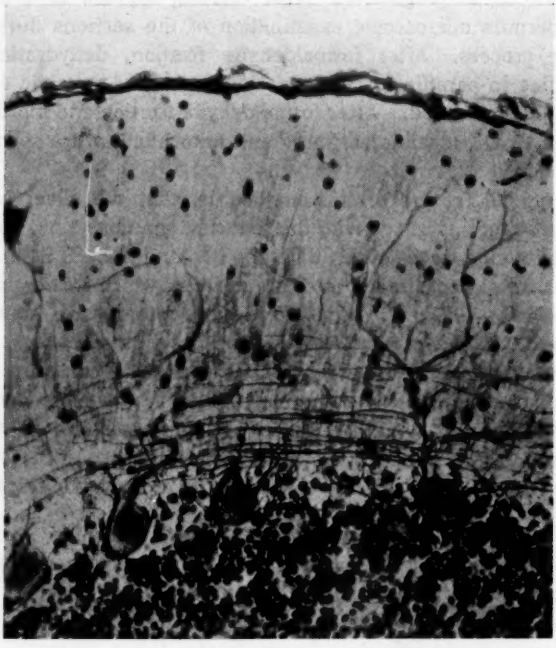

Fig. 1. Photomicrograph of a 12 μ, celloidin section of the cerebellar cortex of a monkey, stained with the silver method described in the text for fine fibers. This tissue was fixed in a formalin-alcohol-acetic-acid mixture (Formol: 10%; Acetic acid: 2%; in 80% ethyl alcohol), dehydrated in higher alcohols for a month, imbedded in celloidin, cut, and mounted on the slide with the clove oil technic. Celloidin was removed with two changes of ether-alcohol and acetone, the slide placed in water, and stained 3 hours with the protargol-Rochelle-salts method at a temperature of 52°C. ×200.

Note the basket fiber endings around the perikaryon of the Purkinje cells.

The method may be used to stain nuclei, fine fibers, and neuronal terminations as follows:

1. Fix tissues, preferably by perfusion with 10% neutral formalin in 1% NaCl, 10% commercial formalin in 1% NaCl, formalin-formic-acid, formalin-alcohol, formalin-acetic-alcohol, Bouin's fluid, etc.

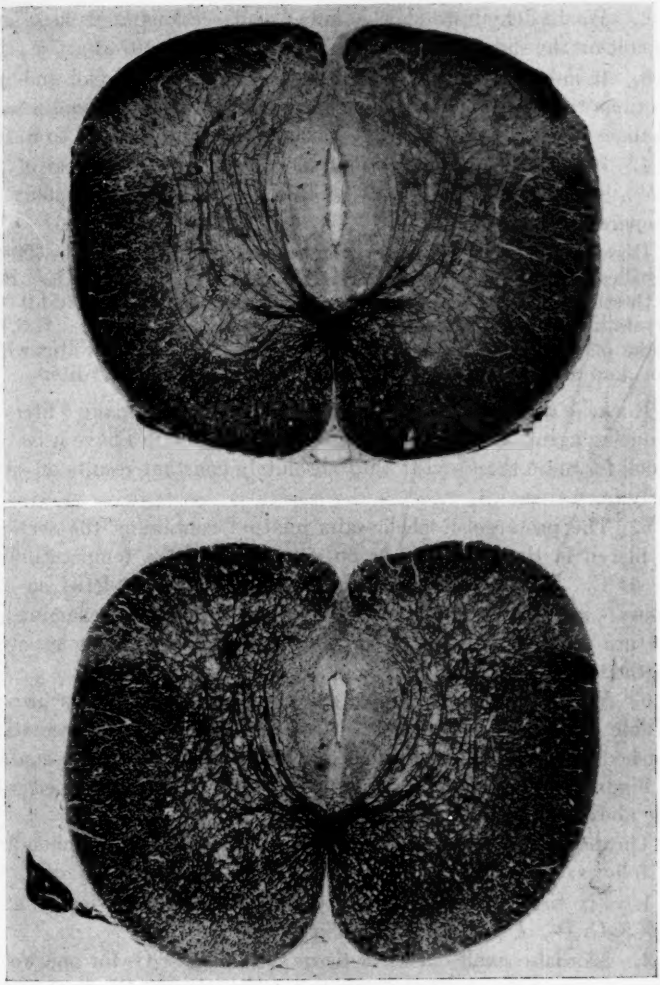

Fig. 2. Photomicrographs of two 20 μ, paraffin-imbedded, transverse sections of the medulla oblongata of the frog at the level of the most caudal end of the IV ventricle. This block was fixed in Regaud's formol-bichromate mixture, and mordanted for a week in 3% $K_2Cr_2O_7$. The tissue was washed in water for 24 hours, dehydrated in increasing concentrations of ethyl alcohol, double-imbedded in 3% celloidin-paraffin, and cut at 20 μ. ×40.

The upper section was stained on the slide with the Weigert method and the lower section was stained on the slide with the silver method described in this paper for myelin sheaths.

Note that the silver-stained section shows more of the finely myelinated fibers than the upper Weigert specimen, and also differentiates the large fiber tracts in the white matter.

2. Wash, dehydrate slowly, imbed, cut sections at 2–20 μ, and mount on the slide; or frozen sections may be cut, 10–40 μ.

3. If imbedded in paraffin, remove paraffin with xylol and run sections to water. If imbedded in celloidin, remove celloidin with changes of acetone and absolute-alcohol-ether, run sections to water.

4. Immerse slides or sections in mixture of equal parts of (a) 0.5% aqueous protargol[2] and (b) Rochelle salts reducing solution, prepared as follows:

Dissolve 64 g. Rochelle salts (potassium sodium tartrate) in 500 cc. distilled water. Boil vigorously. Add 10 cc. of 10% $AgNO_3$. Boil with stirring for at least 5 minutes. Remove the flame. Add 0.3 g. crystalline $MgSO_4$; then while simmering add 0.2 g. K_2S, (U.S.P.) using only the brown, unoxidized portion of one piece. Filter while hot, and add distilled water to make a total volume of 4 liters.

Rochelle salts reducing solution thus prepared will make 4 liters of reducing agent, and improves slightly with age. (We have used one stock for more than a year with absolutely constant results on similarly fixed tissue).

5. The protargol-Rochelle-salts mixture containing the sections is placed in the incubator or on a hot plate, at a temperature of 45–55°C. Staining is progressive, and usually completed in 2–3 hours. Slides may be removed during the process and examined, a feature which makes this procedure relatively unique among silver methods.

6. When staining is completed, usually before there is grossly visible reduction of the silver in solution, the slides are removed, washed in two changes of distilled water, dehydrated and mounted.

Figure 1 is a photomicrograph of monkey cerebellum stained with the above method to demonstrate fine fibers.

The method may be used to stain myelin sheaths and mitochondria, as follows:

1. Fix tissues, preferably by perfusion, with 10% formalin in 1% $K_2Cr_2O_7$, or with 10% formalin in 1% NaCl.

2. Mordant small blocks of tissue in 3% $K_2Cr_2O_7$ for one week. This step may be omitted if the tissue has been in the fixative for more than a week.

3. Wash, dehydrate, imbed, cut sections at 4 to 20 μ, and mount on the slide.

4. Remove imbedding medium, and proceed as above.

Figure 2 is a photomicrograph of the medulla oblongata of the frog, stained to demonstrate myelinated fibers. The upper section

[2]Protargol, strong protein silver (silver albumose), manufactured by the Winthrop Chemical Company Inc., New York, N. Y.

was stained by the Weigert method, and the lower section was stained with the silver method described in this paper for myelin sheaths. Note that it shows even more of the finely myelinated fibers than are demonstrated by the standard Weigert method on the same material.

The staining of the myelin sheaths with this method is specific in intensity for specific fiber-tracts, and aids in their identification when they are followed in successive sections.

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A STAINING RACK FOR HANDLING COVER-GLASS PREPARATIONS

TZE-TUAN CHEN, *Department of Zoology, University of California at Los Angeles*

A porcelain staining rack (Fig. 1) has been devised for handling

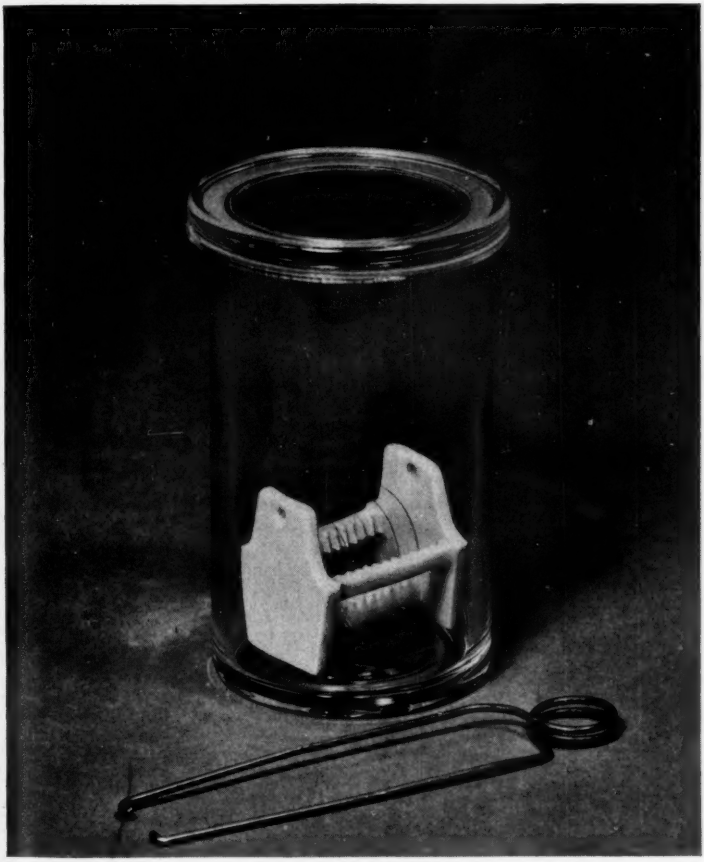

FIG. 1. Photograph of the staining rack in a Stender dish. Wire tongs or handle in front of the dish.

cover-glass preparations. The design is along the general lines of staining racks for slides commonly sold by commercial houses. It

consists of three parallel rods—one at the bottom, two on the sides. These three rods are held together by two end pieces. Each of the rods has twelve slots, thus the rack holds twelve cover glasses. The slots have been arranged to hold the cover glasses some distance apart. Circular cover glasses having a diameter of 22 mm. are most desirable. Each of the two end pieces has a hole near the top for inserting one end of the wire tongs or handle (see Fig. 1) which is used for removing the rack from the stender dish. The staining rack is 40 mm. long and 35 mm. high and is designed, as shown in the figure, to fit the ordinary Stender dishes commonly used in many laboratories.

The obvious advantages of this rack in staining, destaining, and dehydration of cover-glass preparations are: (1) Twelve cover glasses can be handled at once, thereby saving much time. (Larger racks to handle more than twelve cover glasses at a time can be easily designed.) (2) There should be less danger of breakage of cover glasses and of damage to the specimens (especially those near the edges of cover glasses). (3) The staining rack seems more convenient than the so-called "Columbia cover-glass staining dish" designed by Bowen.[1] It is believed that many protozoölogists, parasitologists, some cytologists, and perhaps others should find the staining rack particularly useful.

This staining rack for cover glasses can now be purchased from Arthur H. Thomas Company, West Washington Square, Philadelphia, Pennsylvania, whose courtesy and cooperation is hereby acknowledged.

[1]Bowen, R. H. 1929. A new staining dish for handling cover-glass preparations. Stain Techn., 4, 57–8.

NOTES ON TECHNIC

The Washing Bobber

The small gadget now described has been in use for some years in this laboratory for washing histological specimens. Its extreme simplicity and cheapness is of the greatest advantage when we have to cope with a large number of preparations for class work. It serves its purpose admirably and is just as effective as many other elaborate pieces of apparatus described elsewhere.

The bobber illustrated in Fig. 1 is made as follows. A rectangular piece of perforated zinc is bent round a cylindrical piece of metal or wood of the required diameter. The ends, which may overlap or butt against one another, are soldered. The open tube is based by soldering a small piece of the same metal to it, the rough edges are then trimmed off with shears and the bobber is complete. We have found the most convenient size to be 1½″ high by ¾″ diameter. The tissue is put in the device with a small pencilled label if necessary and the open end is closed with a cork. It is then thrown into a bowl in a sink under a stream of running water from the tap. The bobber moves along the surface and then comes under the jet of water when it suddenly dives and reappears at the periphery of the bowl; this circular motion is repeated over and over again. It was this rapid down and up movement that led our students to christen it "the bobber"; it ensures very thoro washing of the tissue. If necessary, up to twenty bobbers can be accommodated in an ordinary sized bowl without lessening the effects of the washing.—F. W. Gairns, *Institute of Physiology, University of Glasgow, Scotland.*

Clarite in Embedding Paraffin for Thin Sections

During the course of preparing slides of *Triturus* larvae of 15 mm. and less, the writer had to work in a room too warm for sections in ordinary paraffin mixtures. As the embedding oven used was employed by a number of other people, it was not convenient to embed in very high melting-point paraffin. It was discovered that addition

of small amounts of clarite (Nevillite V) provided a much harder block and by varying the amounts added, sections down to 2–3 μ might be cut at ordinary room temperature. With a mixture of 90% paraffin 53°C. m.p., 5% bleached beeswax, and 5% clarite, by weight, satisfactory sections may be made in a warm room. With this mixture, however, the ribbons normally become charged with electricity more than ordinary ribbons so that a spark-coil device such as described by Blandau[1] should be used.—WILLIAM WEHRLE[2], *Berkeley, Calif.*

[1]Blandau, R. J. 1938. A method of eliminating the electrification of paraffin ribbons. Stain Techn., **13**,139–141.

[2]Formerly employed by the U. S. Works Progress Administration. Aid from this Administration (Official Project 65–108–113, Unit C-1) is hereby acknowledged.

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEW

POLYAK, S. L. **The Retina.** 7½ x 10½ in. 608 pp. + 114 pp. of plates. Cloth. University of Chicago Press. 1941. $10.00.

The principal object of this elaborate book is the elucidation of the complex structure of the retina and the interpretation of the basic visual functions in terms of structures of the visual organs. The book is divided into five parts and thirty-four chapters. Part I deals with the histological technic used by the author. The Golgi-Cox method (with $HgCl_2$ instead of $AgNO_3$) was used because of greater uniformity of impregnation. The other procedure used was the methylene blue stain of Ehrlich by injection of the dye into the vitreous of living animals. The disturbing factor of both methods is their irregularity. The methods do not stain all the elements of a tissue simultaneously but rather in a haphazard way, picking up here and there one or another of its particular constituents. Conclusions concerning morphological interrelationships as well as functional mechanisms are not always drawn with extreme care, as should be done because of the uncertainty of methods used. Parts II and III deal with a survey of investigations from ancient to contemporary times. The most valuable part of this work seems to be the minute description of the various nerve cell elements of the retina with their synapses (part IV). Part V deals with the functional interpretation of retinal structures. The neuroglia is considered an insulator of neurons and their expansions. Small asteriform elements confined to the territory of the seventh layer are recognized as microglia. The theory of a continuous glial syncytium is called "a phantasmagoria fathered by prejudice and mothered by lack of experience". The author is strongly opposed to the doctrine of the nerve nets (neurencytium) and adheres to a somewhat modified neuron doctrine. A number of illustrative photomicrographs and drawings present a very valuable addition to the text.—*Gabriel Steiner.*

MICROSCOPE AND OTHER APPARATUS

COOK, F. W., SATTERFIELD, G. H., and RALEIGH, M. A. **Multiple tissue washer and processing assembly. Combination apparatus facilitating the preparation of tissues for paraffin section.** *J. Lab. & Clin. Med.*, **27**, 673–9. 1942.

The tissue washer consists of a deep, 20-gauge, 6-qt. aluminum pudding pan perforated with 27 holes joined to short copper tubes with solder, each holding a 7-in. length of rubber tubing provided with a Hoffman clamp. Thus a reservoir of water from a single tap is provided for from 1 to 27 assemblies. The washer is durable, inexpensive, easily constructed, requires a small space and gives a regulated flow for each tissue container.—*John T. Myers.*

CRABB, EDWARD D. **A multicolored wax light filter and dissecting chamber holder for microscopes.** *Rev. Sci. Instruments*, **12**, 154. 1941.

The base for this combination light filter and watch glass holder is a synthetic wax, known commercially as "Flexowax C," which is colored with oil-soluble dyes. The wax and six "Glycolors" (black, blue, brown, green, red and yellow) may be obtained from Glyco Products Company, 148 Lafayette Street, New York.

In combining the colors a small quantity of Flexowax C was melted and the dye added until a deep color was obtained. After cooling, the plastic wax was rolled into small balls and stored until needed in separate shell vials. Additional colors

were obtained by kneading small pieces of two or more differently colored "marbles" together to form pellets.

The simplest form of four-colored filter may be made by placing four pellets of colored wax, or three of colored and one of uncolored, on the convex side of a watch glass in the form of a square surrounding the center, then turning the watch glass upright and pressing it firmly onto a glass plate. The wax serves as readily as a background for direct illumination, as a filter for transmitted light.

The most satisfactory filters for use with higher magnifications were made as follows: Pellets of colored wax were flattened to the desired thickness by being compressed between two glass plates wet with alcohol. The thickness was controlled by two strips of cardboard between the plates. A triangular piece was cut from each flattened pellet and properly spaced around the summit of the convex surface of a small watch glass with the apices directed inward. After the alcohol had evaporated, the watch glass was attached to a glass slip by compressing the wax triangles.—*A. B. Dawson.*

WOLFSON, C. **An improvised inverted microscope.** *J. Lab. & Clin. Med.*, **27**, 809–11. 1942.

An inverted microscope for use in photomicrography, tissue culture and micrurgy may be prepared as follows: Affix the arm and body of a standard microscope (Spencer Model 68 was used) to a cast iron base. Replace the nosepiece with a prism chamber (3¼ in. long with an internal cross section of 1.0 in., made of ⅛ in. sheet aluminum). Screw it into the body tube by means of a threaded collar and lock with a lock nut. Drill two ⅝ in. holes in the top of the chamber, one concentric with the body tube and the other with the objective. Place two right-angled prisms in the chamber, fitting them snugly, and fasten them by screws thru the floor of the chamber and by plates which close the ends. Mount the three objectives on a brass plate, each threaded into a collar. Make the plate movable between two guides perpendicular to the length of the prism chamber. Fit a threaded machined band about the collar and fix it with a set screw. Each band should have a machined depression which receives a spring-backed pin when the objective is centered. Support the condenser and the light source (an 18 v. toy train lamp) on a sturdy upright mounted on the base (a 5½ in. length of ¾ in. square brass rod with a brass plate affixed to its upper end by means of a centered screw). Fix a mechanical stage to the iron base. Use the entire condenser with the rack and pinion (Spencer Model 3 Research Microscope). Fasten a thin upright to the condenser for supporting a cover slip to serve as a stage between the condenser and the objective. This equipment is readily available and is inexpensive.—*John T. Myers.*

PHOTOMICROGRAPHY

KOCH, WALTER. **Increasing the depth of focus in photomicrography by incident light.** *J. Roy. Micr. Soc.*, **61**, 86–7. 1941.

The depth of focus, using incident illumination, can be increased by inserting a stop immediately above the object glass. Three thin circular plates are prepared, each the diameter of the fixed diaphragm of the object glass. In the center of each plate is a hole, 1, 2, and 3 mm. in diameter, respectively. A series of 5 photographs is made of a stage micrometer viewed thru the microscope by transmitted light. The first is made with the micrometer horizontal; the second with one end of the micrometer lifted about 2 cm.; the third, fourth, and fifth are made with the micrometer in the oblique position, each stop being inserted in turn above the object glass. The depth of focus is determined by counting with the aid of a magnifier the number of division marks which appear sharp on each photograph. The narrower the stop the more marks will appear sharp; however, with increased narrowness there is a progressive loss of resolving power.—*C. E. Allen.*

MICROTECHNIC IN GENERAL

BRUG, S. L. **The use of cajuputin oil in microscopy.** *Trans. Amer. Micr. Soc.*, **61**, 84–5. 1942.

Cajuputin oil is a useful substitute for xylol in wet climates because it will absorb small amounts of water without clouding. Apparently it was not tried as a solvent for balsam.—*Virgene Kavanagh.*

ELLINGER, P. Fluorescence microscopy in biology. *Biol. Rev. Cambridge Phil. Soc.*, **15**, 323–50. 1940.

An historical review of fluorescence microscopy is given, starting with the work of Kohler, in the development of microscopy with ultra-violet light. In a section on technic the principles involved are discussed and the type of apparatus necessary is described. Attention is given to recent advances in this field of technic, especially in regard to new sources of ultra-violet light and to the use of flourescent dyes to increase the visibility of objects in such light. A section on results follows which is divided into the following sub-headings: examination of spontaneous fluorescence of animal and plant tissues; fluorescence microscopy in bacteriology; results of intravital microscopic studies. The article ends with a summary and a good bibliography, thus providing a useful introduction to a study of the field covered.—*H. J. Conn.*

MEDAWAR, P. B. The rate of penetration of fixatives. *J. Roy. Micr. Soc.*, **61**, 46–57. 1941.

Plasma is prepared from a cockerel by bleeding from the carotid artery, spinning the blood, and storing the supernatant plasma in waxed test tubes on ice. For experimental purposes it is coagulated by warming to 37° C., or by adding a trace of a tissue extract in saline, in short glass tubes of constant bore sealed at one end. These tubes are stood in stoppered vessels containing a volume of fixative at least 30 times the volume of the plasma. The fixative is periodically renewed. For fixatives which precipitate proteins, the rate of fixation is measured by the distance, at definite times, of the surface of the plasma from the boundary formed at the level where the reagent is of a concentration sufficient for precipitation. For other fixatives, special indicators are added to the plasma: for potassium dichromate, methylene blue; for acetic acid, brom thymol blue; for osmic acid, 1% pyrogallic acid; for formaldehyde, phenylhydrazine hydrochloride in saturated aqueous solution; for iodine, starch solution. Temperature and the concentration of the fixative remaining constant, the distance (x) penetrated by the wave front (boundary of precipitation) depends upon the following function of the time of fixation (t):

$$x = K\sqrt{t}$$

K is called the coefficient of penetration. Its value was determined for each of various simple fixatives, in the concentrations in which these are used in standard fixing fluids, as well as for the combined fluids themselves. In the table of results given, K is highest for 25% acetic acid, lowest for 1% tannic acid.—*C. E. Allen.*

PENNY, S. F. A substitute for cover glasses in mounting pathological specimens. *Can. J. Med. Techn.*, 2 (No. 2, March). 1940.

Due to rise in price and lack of German supply, the author tried various thicknesses of cellophane, plastocele, and mica, using gum dammar as mounting medium. All proved unsatisfactory. Attempts were made to find a transparent plastic material which could be applied in liquid form, drying with a hard, smooth surface. Clear Duco was fairly satisfactory, but a new quick-drying clear lacquer has proved the best material so far. The lacquer used is C.I.L. Special Slide Lacquer XB 6965–(S.R. 2452; I.R. 23–710). Retail price, January 1940, was $7.50 per gallon, obtained from Paint and Varnish Division, Canadian Industries, Ltd. This lacquer has an advantage over Duco in that it is soluble in xylol, and may be applied to sections direct after removal from xylol. Apply the lacquer by brushing it lightly over the section and the surrounding slide with a camel-hair brush about 1.5 cm. in width. The sections are then allowed to dry at room temperature and are ready to be examined after 15 minutes. The lacquer surface smoothes out and brush marks disappear. It has remained clear after exposure to light for several months. Under high-power oil immersion the cell detail is slightly hazy. The mixture index was estimated to be 1.4835, and this is reputed to be the nearest index to glass of any of the quick-drying lacquers. If a high-power dry objective with compensating collar is used, the refractive error can be corrected and cell detail becomes quite clear.—*S. G. L., in J. Roy. Micr. Soc.*, **61**, 105.

PUSEY, M. A. **Methods of reconstruction from microscopic sections.** *J. Roy. Micr. Soc.*, **59**, 232–44. 1939.

Drawings are made from a series of sections (not less than 13 μ in thickness) and then superimposed in the original order in a tracing box lighted from above and below, the paper sheets bearing the drawings being made transparent with xylene. At three points in a triangle, "guide holes" are made thru the whole pile of drawings. In preparing a "contour picture", the position of the guide holes is noted on the tracing paper; then, orienting the drawing by means of the guide holes, the outlines of the selected structure or structures are traced from the first drawing. Later drawings in proper order replace the first, and those parts of each drawing are traced which project beyond previous drawings. By appropriate use of dotted and heavy lines the contours of the structure or organ in question are adequately shown. A method is outlined also for securing a "projection reconstruction" at right angles to the planes of the original sections. Many detailed recommendations are offered with regard to the trimming of the block in which material is imbedded, the cutting and mounting of sections, outlining of the sections on the slide with a black paint, and the preparation of drawings (using a microprojector rather than a camera lucida).—*C. E. Allen.*

DYES AND THEIR BIOLOGICAL USES

BARNES, W. A. **Effect of Congo red on plasma prothrombin.** *Proc. Soc. Exp. Biol. & Med.*, **49**, 15–9. 1942.

The hemostatic action of Congo red may be explained in some cases by its vitamin-K-like action in raising the level of plasma prothrombin. This action was tested in mice, the level of plasma prothrombin of which had been lowered by feeding mineral oil.—*M. S. Marshall.*

CANNON, H. GRAHAM. **On chlorazol black E and some other new stains** *J. Roy. Micro. Soc.*, **61**, 88–94. 1941.

The author introduced chlorazol black E into microscopic work in 1937, and now calls further attention to it, re-emphasizing its special usefulness. He states that in some of his preparations he noticed a faint pink, which proved to be due to another dye present. This dye was isolated for the author by the Imperial Chemical Industries, and he has named it "lignin pink" because of its affinity for lignin in staining plant tissue; it is a mono-azo dye. A useful double stain for plant sections consists of 0.5 g. each of chlorazol black E and of lignin pink in 100 ml. water, with a 20–30 minute staining period.

A further series of new stains has been developed as a result of these studies and are now on sale by the British Drug Houses, Ltd. The author names these dyes as follows: "Hickson purple", a disazo dye of unusual chemical structure; of use in differentiating blood cells in tissues. "Marshall red", a disazo dye belonging to the J-acid urea class of direct dyes; of use in combination with "victoria green G". "Beyer brown", a disazo dye of the benzidine class; a good tissue stain, producing results very similar to Ehrlich's hematoxylin. "Victoria green G", a trisazo dye of the benzidine class of direct cotton dyes; stains distinctly differently in aqueous and in alcoholic solution, and can give good double staining effects with "Hickson purple" or "Marshall red", altho these dyes are all essentially nuclear stains. "Manchester blue", a disazo direct cotton dye of the benzidine class. "Owen's blue", a dye similar in constitution to the last mentioned; a very powerful stain with considerable metachromatic effect on plant tissue.

The double staining methods recommended call for 20 min. in saturated aqueous Marshall red or 10 min. in saturated aqueous Hickson purple, followed by 30–60 min. in saturated Victoria green G in 70% alcohol, with a rinse in 90% alcohol, dehydration and mounting. Either combination gives good double staining in animal tissue.—*H. J. Conn.*

CRAIG, JOHN J., and CASS, W. E. **Derivatives of aminoisoquinolines.** *J. Amer. Chem. Soc.*, **64**, 783. 1942.

The procedures for the preparation of several compounds from isoquinoline and preliminary pharmacological tests on the sulfanilyl derivatives are described. The compounds prepared are 4-bromo-, 4-amino-, and 5-aminoisoquinolines and

acetyl, benzoyl, acetylsulfanilyl and sulfanilyl derivatives of 1-, 4-, and 5-aminoisoquinolines. Results of the pharmacological tests show that 5-sulfanilamidoisoquinoline exhibits toxic symptoms in mice in dose levels of 5, 10 and 20 mg. per 20 g. of animal body weight, while the 1-, and 4-sulfanilamido derivatives do not. In tests for antistreptococcal efficacy in mice, protection was obtained with 1-sulfanilamidoisoquinoline comparable with a sulfadiazine standard; protection by the 4- derivative was somewhat less; the 5- derivative was ineffective.—*A. P. Bradshaw.*

DAVIS, EDWIN. **Aniline (triphenylmethane) dyes in the treatment of Hunner ulcer, preliminary report.** *J. Urol.*, **46**, 899. 1941.

The alkylated triamino-triphenylmethane dyes, gentian violet, methyl violet, ethyl violet and crystal violet, were found to inhibit the growth of *Escherichia coli* and *Staphylococcus aureus* in dilutions of 1:10,000 to 1:1,000,000 in urine of pH 5.5 and 7.5. These dyes were usually inhibitory at higher dilutions in urine of pH 7.5 than of pH 5.5.

For the clinical trials, purified methyl violet B (American Aniline Products Company) and crystal violet (Du Pont) were mainly used. For installation, the concentrations used were 1:100 to 1:20,000, while for irrigation, they were 1:10,000 to 1:20,000. Local treatment of the submucous ulcer with these dyes usually resulted in the following sequence of events: an irritating reaction of 1 to 10 days duration, a period of relief averaging 5½ months and then a relapse.—*L. Farber.*

FREDERICK, WILLIAM G. **Estimation of small amounts of antimony with rhodamine B.** *Ind. Eng. Chem., Anal. Ed.*, **13**, 922. 1941.

This paper reports the result of an investigation of the reaction between quintivalent antimony and rhodamine B, and describes a colorimetric procedure for the determination of antimony encountered in tissue and air, based upon this reaction. Rhodamine B reacts with quintivalent antimony in hydrochloric acid solution or sulfuric acid solution containing chloride ion, to form a red, water-insoluble stable compound, soluble in organic solvents. The excess reagent is destroyed with bromine, the rhodamine B complex put into solution by addition of alcohol, and the amount present estimated colorimetrically in a Duboscq colorimeter. Practically all acid radicals except chloride and sulfate interfere, as do arsenic, iron, and certain metals of the dithizone group. The latter interference can be avoided by preliminary extraction with dithizone. The method will detect as little as 0.1 γ of quintivalent antimony in 5 ml. of solution, and is rapid, inexpensive and specific. The extract procedure is described for the concentration range 0.1 to 300 γ of antimony. (There are many precautions given in the original article and it would be advisable to refer to it before attempting the estimation of antimony by this method.)—*A. P. Bradshaw.*

GREEN, J. H. **Sodium salt of quinizarin-6-sulfonic acid as an acid-base indicator.** *Ind. and Eng. Chem., Anal. Ed.*, **14**, 249. 1942.

This indicator can be used for colorimetric determinations of pH over the range from 7 to 11.5 in the titration of strong acids with strong bases, or of weak acids with strong bases. The indicator has two color changes, yellow to pink (pH 7.1–9.1) which is sharp, and pink to blue-violet (pH 9.1–11.5) which is gradual. The author found that in titrating acids with bases the color change was sharper when using the sodium salt of quinizarin-6-sulfonic acid than when using phenolphthalein, and in titrating bases with acids the quinizarin indicator gave a sharp color change, whereas phenolphthalein could not be used so efficiently.—*A. P. Bradshaw.*

HENDERSON, H. J., and LONG, E. R. **Accumulation of chlorophyll pigments in visceral organs and their elimination.** *Proc. Soc. Exp. Biol. & Med.*, **48**, 438–9. 1941.

Chlorin-e and rhodin-g were eliminated slowly from the tissue of rabbits after intravenous injection. Most of that deposited in the spleen and bone marrow during a month of inoculations was still present in phagocytic cells three months later. Elimination from the liver was somewhat greater. The deposit in hemopoietic organs had no evident effect on their function.—*M. S. Marshall.*

LEHR, DAVID, and ANTOPOL, WILLIAM. **Specific morphology of crystals appearing in the urine during administration of sulfanilamide derivatives.** *Amer. J. Clin. Path.*, **12**, 200–9. 1942.

Sulfanilamide derivatives are excreted in the urine as "impure" crystals with the following specific morphology: Sulfathiazole crystals are amber green and are shaped like dumb-bells, rosettes or hexagonal platelets. Sulfadiazine is excreted in acetylated form with crystals of amber green shells and excentrically bound shocks of wheat; or in free form, with dark green globular and chestnut burr crystals. Sulfapyridine crystals resemble whetstones, arrowheads or coarse, centrally waisted sheaves. Sulfaguanidine crystals appear as oblong rectangular plates bulging slightly in their long axis.—*H. Gordon.*

MICHELSON, LOUIS. **An improved polychrome methylene blue eosinate.** *J. Lab. & Clin. Med.*, **27**, 551–3. 1942.

The stain is prepared as follows: Dissolve 1 g. of methylene blue chloride (certified) in 100 cc. of *N*/100 NaOH (carbonate free) in a 500 cc. glass-stoppered bottle. Heat in a water bath for 2½ hr. at 55° C. Shake vigorously for 1 min. at half-hour intervals. Add 1 g. of NaBr, heat again for 2½ hr., cool and filter. Add 60 cc. of 1% aqueous eosin Y and mix with a stirring rod. Add more eosin in 5 cc. amounts till the solution has a reddish tinge. Let stand 18–24 hr. and filter. Dry completely at 37° C. Store in a glass-stoppered bottle. Dissolve 0.1 g. in 600 cc. of absolute acetone-free methyl alcohol. Let stand 2–3 days and filter.—*John T. Myers.*

ROBLIN, RICHARD O., JR., WINNEK, PHILIP S., and ENGLISH, JACKSON P. **Studies in chemotherapy. IV. Sulfanilamidopyrimidines.** *J. Amer. Chem. Soc.*, **64**, 567. 1942.

The preparation and properties of a series of sulfanilamidopyrimidines related to sulfadiazine is reported. With the exception of 2-sulfanilamido-4, 6-dimethylpyridine, none of the compounds in this series showed the same high degree of absorption and chemotherapeutic activity against experimental streptococcal and pneumococcal infections, as sulfadiazine. Because of the unfavorable influence of a methyl group on toxicity as evidenced by sulfamethylthiazole, an extensive investigation of toxic properties would be required before a true evaluation of its utility could be given. 5-Sulfanilamidopyrimidine was active but was not as effective as sulfadiazole. The 2-amino derivative was found to be only slightly active. When the sodium salt of this compound was administered subcutaneously it produced a much better therapeutic effect by increasing the blood levels. 2-Sulfanilamido-4-methoxypyrimidine was less active than the corresponding 4-methyl compound.

4-Sulfanilamidopyrimidine was described as inactive on the basis of *in-vivo* experiments published in a previous paper. 2-Sulfanilamidopyridine was found active against experimental infections in white mice. Since such a marked distinction between the 2- and 4-isomers seemed unlikely, the 4-isomer was retested *in vitro* and found to show as great a bacteriostatic effect as sulfadiazine. The authors call attention to pitfalls which might be involved in the use of *in vivo* data as a basis for attempts to correlate chemical structure with chemotherapeutic activity.—*A. P. Bradshaw.*

WHITBY, G. S. **Dyes and dye chemistry fight disease.** *Textile Colorist*, **64**, 119. 1942.

This interesting review brings out the historical development of chemotherapy and the direct discovery of better colorless drugs for use as chemotherapeutic agents arising from the study of the use of dyes for this purpose. Trypan red, in the treatment of the special disease Mal de caderas, led ultimately to (a) the colorless remedy Germanin, for human sleeping sickness, (b) the arsenical drugs, such as Salvarsan for syphilis. From Prontosil, a dyestuff by virtue of its azo group, the new class of synthetic drugs, the sulphonamides, has been developed. Furthermore, leading to the discovery of Prontosil was (a) the discovery that the yellow azodyl, chrysoidin, had appreciable antiseptic powers *in vitro*, altho lacking *in vivo*; (b) the discovery of the bacterial properties of certain sulphonamides; and (c) the successful employment as an internal antiseptic of an azo dye, pyridium.—*A. P. Bradshaw.*

ANIMAL MICROTECHNIC

ANONYMOUS. **Hastings' Stain. Method of Preparation.** *J. Amer. Med. Assoc.*, **118**, 936. 1942.

In answer to a query concerning the preparation of Hastings' stain for blood films in determining the opsonic index in undulant fever, the modification of Wright's stain known as "Hastings' stain" for blood films is described as follows: "It was described by T. W. Hastings (*J. Exp. Med.*, **7**, 265, 1905) and differs from the stain described by Wright in that the polychrome methylene blue is neutralized with acetic acid and, when mixed with the eosin solution, a small amount of fresh methylene blue solution is mixed with it." I. F. Huddleson specifies this stain in a recent publication and says that it can be purchased from Hartman-Leddon Co., Philadelphia, the only reliable source he has found. There is no information available to the Biological Stain Commission to indicate that there is any essential difference between this stain and the one this company sells as Wright's stain.—*Elizabeth F. Genung.*

BAKER, JOHN R. **A fluid for softening tissues embedded in paraffin wax.** *J. Roy. Micr. Soc.*, **61**, 75–8. 1941.

A mixture of 9 parts of 60% alcohol and 1 part of glycerin is recommended for mammalian liver, pancreas, kidney, and other tissues. The block containing the imbedded material is soaked in the softening fluid after a surface of the tissue is fully exposed by a razor cut. Rate of penetration varies with the tissue and with the fixative used. Soaking overnight is generally satisfactory.—*C. E. Allen.*

COOKE, JEAN V., and BLATTNER, R. T. **Trypan-blue vital staining in studies of virus lesions on chorio-allantoic membranes.** *Amer. J. Path.*, **18**, 163–7. 1942.

The authors describe the following new application of trypan-blue for vital staining in which virus foci grown on chick chorio-allantoic membranes are selectively outlined by the dye: When the membrane is ready for examination, following inoculation and incubation, place 1 cc. of a 0.5% aqueous suspension of trypan-blue (dye content and source not stated) directly upon the membrane thru the window in the shell. It was found that the stain could be best applied to the membrane with a No. 15 DeVilbiss atomizer. After application of the stain close the shell window and place the egg in the incubator for 10 to 30 min., the incubation time depending upon the age and extent of the lesion. At the end of the period of incubation remove the membrane in the usual manner, wash gently in physiological saline to remove the excess of trypan-blue, and fix flat in a 4% solution of formaldehyde for a few minutes. After fixation drain the excess formalin from the membrane and mount in warm glycerin jelly on a large glass slide. For mounting, melt the glycerin jelly and drop it on the membrane until the latter is covered. Flame the slide gently and add a warm cover slip, applying gentle pressure to the cover slip until the jelly sets. When hardened, remove the excess jelly from around the edges of the cover slip and seal the edges with clear Duco cement thinned with acetone.—*J. O. Foley.*

FETTERMAN, GEORGE H. **A simple and reliable method of staining spermatozoa.** *Amer. J. Clin. Path., Tech. Suppl.*, **6**, 9. 1942.

Abnormal spermatozoa can be identified in smears of fresh seminal fluid by the following method: Air-dry the smear; fix 3 min. in 10% aqueous formalin (4% formaldehyde); stain 1 min. with Harris hematoxylin; wash; dry.—*H. Gordon.*

HARTZ, PHILIP H. **Routine staining of the beta cells of the islets of Langerhans with Masson's tetrachrome stain.** *Arch. Path.*, **33**, 541–2. 1942.

The β cells of pancreatic islet tissue may be stained specifically by the following method: Fix thin slices of pancreatic tissue 12–24 hr. in equal parts Bouin's fluid and saturated aqueous solution of $HgCl_2$; embed in paraffin; cut 6 μ thick; deparaffinize; remove mercury precipitate; stain in Larson and Levin's modifica-

tion of Masson's tetrachrome stain (*Arch. Path.*, 29, 272, 1940), substituting light green with fast green; dehydrate; clear; mount in balsam. Result: the β cells contain red granules.—*H. Gordon.*

HASS, GEORGE M. **Studies of Cartilage. III. A new histochemical reaction with high specificity for cartilage cells.** *Arch. Path.*, 33, 174–81. 1942.

Cartilage cells react in a specific manner to the following technic: Section fresh blocks of cartilage at 15–20 μ on the freezing microtome; wash; place for 45 min., at room temperature in a mixture of 10 ml. 0.5 molar HCl, 4 ml. 0.04 molar crystal violet, and 1 ml. 0.2 molar $NaNO_2$; add 7–8 ml. 0.5 molar NaOH, till the solution becomes slightly fluorescent and forms a slight precipitate after 10–20 min.; pour ether over surface of solution; withdraw and wash sections; float on slide; blot with filter paper; stain with hematoxylin; mount in glycerogelatin.

Results: a positive reaction is indicated by aggregations of spherical or discoid, refractile yellow granules, 1–3 μ in diameter, occurring in the cytoplasm of cartilage cells. The nature of the reacting cytoplasmic component is unknown. Infantile cartilage reacts more uniformly than adult cartilage. Noncartilaginous tissues do not react.—*H. Gordon.*

HOLMES, WILLIAM. **A new method for the impregnation of nerve axons in mounted paraffin sections.** *J. Path. & Bact.*, 54, 132–6. 1942.

The method is applicable after formalin fixation and uses $AgNO_3$ as the impregnating agent. It gives a uniform impregnation of the axons of medullated and non-medullated nerve fibers, and in preliminary tests has given good results with material from the central nervous system.

The procedure is as follows:

Fix specimens upon removal in a mixture of 40% neutralized formalin, 15 cc., and 0.8% aqueous NaCl, 85 cc. Transfer tissue directly to 95% alcohol, pass thru absolute alcohol, and prepare paraffin sections as usual. Attach sections to albuminized slides, dry 24 hr. at 37°; 15 μ is a good thickness if no counterstain is to be applied. Soak the slides for 48 hr. at 37° C. in a mixture of xylene, 30 cc., and glacial acetic acid, 70 cc. Take down to distilled water thru graded alcohols. Impregnate with a mixture of 0.01% aqueous $AgNO_3$, 100 cc., and concentrated ammonia, one drop for 5–24 hr. at 37° C. This solution can be used only once. In subsequent stages the slides are treated individually. Rinse in distilled water for 5 sec. Reduce in either a strong or weak reagent. Preparation of the strong reducer is as follows: anhydrous Na_2SO_3, 5 g. (or 10 g. of hydrated); $NaHSO_3$, 2.5 g.; water, 100 cc. To this add immediately before use 0.5 g. amidol. To prepare weak reducer: dissolve 1 g. hydroquinone, 5 g. anhydrous Na_2SO_3, in 100 cc. water. Place slides for 30 sec. in the reducer. Wash slides by violent agitation in running water 10–15 sec., then in distilled water 5 sec. Tone sections for 3–5 min. in 0.2% of either yellow or brown gold chloride. If the yellow salt is used, add 4 drops of glacial acetic to each 100 cc. of solution. Agitate slides 5 sec. in distilled water. Reduce in 2% aqueous oxalic acid for not more than 10 min. Control appearance of nerve fibers under the microscope. Rinse slides briefly in distilled water. Place in 5% aqueous $Na_2S_2O_4$ for 5 min. If the solution becomes cloudy the oxalic acid was not removed by the previous rinse. Wash slides thoroly in distilled water; counterstain if required; clear and mount.

The stronger reducer brings out finer axons but stains other structures deeper. Preliminary results indicate that the method may be applicable to all parts of the nervous system and to material fixed in other fixatives.—*S. H. Hutner.*

KOLIN, MORRIS. **Technic for sectioning soft bones and hard tissues by celloidin and paraffin methods.** *Arch. Path.*, 33, 86–7. 1942.

The staining qualities of soft bones and hard tissues are protected and their sectioning made easy by the following technic: Fix 24 hr. in 1:9 aqueous formalin (4% formaldehyde); wash 3–4 hr.; soften 24 hr. in 5% aqueous glacial acetic acid (C. P.); wash 24 hr.

For celloidin embedding: dehydrate 1 hr. in 80% alcohol; 1 hr. in acetone (C. P.); 3–6 hr. in oil of clove (U. S. P.); infiltrate 24–48 hr. with 8% pyroxylin in equal parts ether and absolute alcohol; embed in 12% pyroxylin.

For paraffin embedding: dehydrate 12 hr. each in 80%, 95%, and absolute alcohol; infiltrate 1½ hr. in chloroform; 2 hr. at 52° C. in chloroform, 1 part, with paraffin, 2 parts; 2 hr. in pure melted paraffin; embed in paraffin.

Routine hematoxylin (Harris) and eosin staining methods are recommended after both types of embedding.—*H. Gordon.*

PEARSON, GRACE A. **Eosin Y for tissues.** *Amer. J. Clin. Path., Tech. Suppl.*, **6**, 16, 1942.

A rich, fast-staining eosin Y may be obtained as follows: Dissolve 2.5 g. eosin Y (National Aniline and Chemical Co.), in 500 ml. water; add, drop by drop, 4 cc. concentrated HCl; allow precipitate to settle; decant supernatant fluid; wash precipitate 6–8 times in 500 ml. distilled water; filter; dry in oven; dissolve in 200 ml. 95% alcohol; use full strength. Protect the dye by immersing slides in 95% alcohol before staining in the eosin.—*H. Gordon.*

RITCHIE, GORTON. **A simple iron hematoxylin method for tissues.** *Amer. J. Clin. Path., Tech. Suppl.*, **6**, 10. 1942.

Designed to fortify the action of weak hematoxylin for Van Gieson's stain, the method is as follows: Mordant sections 10 min. in 2–4% aqueous $FeNH_4(SO_4)_2 + 12\ H_2O$ (violet crystals); wash well in tap water; stain 2–5 min. with hematoxylin (Harris or Delafield).—*H. Gordon.*

STOVALL, W. D., and PESSIN, S. B. **Problems in the laboratory diagnosis of rabies.** *Amer. J. Pub. Health*, **32**, 171–4. 1941.

The authors find eosin-methylene-blue the most satisfactory stain for demonstrating Negri bodies in routine diagnostic work, both for paraffin sections and for smear preparations. It is important that staining solutions of low pH be used. A 1% eosin Y in 95% alcohol maintained at pH 6 stains Negri bodies a pale pink color; at pH 3 the large and small Negri bodies stain a uniform deep red. The counterstain is methylene blue adjusted between pH 5.0 and 6.0. When eosin and methylene blue are maintained at the optimum pH, the cytoplasm of the nerve cells takes a diffuse pale blue color and the body of both the small and large Negri bodies a deep red. The inner structure of the Negri bodies appears as dark blue granules. The "lyssa bodies" are a homogeneous red color.—*M. W. Jennison.*

WILSON, WILLIAM L. **A quick easy method of preparing tissues for microscopic examination by combining the technics of Nelson and Terry.** *J. Lab. & Clin. Med.*, **27**, 537–42. 1942.

The following method is quick, easy and inexpensive: Dissolve 3 g. of crystalline egg albumin in 10 cc. of tap water. Place a cellophane container in a dish or beaker and put the block of tissue in it. Fill the cellophane container with albumin solution and pour the fixing fluid (40% formaldehyde) around it. Let stand 2–8 hr. depending on the temperature (140–170° F.). Hold the block in running water for a few seconds, and peel off the cellophane, stain with Terry's neutralized polychrome methylene blue; mount and examine. This technic is particularly useful in the office of surgeons and gynecologists.—*John T. Myers.*

PLANT MICROTECHNIC

BHADURI, P. N. **Improved smear methods for rapid double staining.** *J. Roy. Micr. Soc.*, **60**, 1–7. 1940.

The following changes are suggested in the Feulgen-light-green method previously described: For root-tip smears, treat roots 3–24 hr. (according to species) with 0.1% colchicine or 1% chloral hydrate. Fix tips in Levitsky's fixative (6:4) for 2–3 hr.; rinse in water; soak 5 min. in warm water; hydrolyze in *N* HCl 20–30 min. at 60° C.; and stain in decolorized basic fuchsin solution 20–30 min. Smear the tissue in 45% acetic acid between cover glass and slide; remove the cover glass in acetic alcohol (1:1), and leave the smear in this solution 10 min. Then pass thru 2 changes of 80% alcohol, 5 min. each. Mordant for 45–60 min. in a clear saturated solution of Na_2CO_3 in 80% alcohol, wash in 3 changes, 5 min.

each, of 70% alcohol, bring to 90% alcohol and stain 10–15 min. in light green. Differentiate the stain in 80% alcohol with a trace of Na_2CO_3, followed by 90%, 95%, and absolute alcohol, and clear in graded xylol-alcohol. Mount in "Sira" medium.

A more rapid method for general cytological observation is outlined, involving fixation in modified Carnoy (3:1:1) and omitting the light green stain. Modifications are suggested for preparations of pollen mother cells and pollen grains.—*C. E. Allen.*

CHEN, H. K., and THORNTON, H. G. **The structure of "ineffective" nodules and its influence on nitrogen fixation.** *Proc. Roy. Soc.* (*B*), **129**, 208–29. 1940.

For studying distribution of starch, material was fixed in Allen's modification of Bouin's solution (Allen's P.F.A. 3) and occasionally in Flemming's (McCoy 1929). Slides were treated with a mordant of 2% aqueous solution of tannin for 12 hr., stained 2 min. with 1% aqueous crystal violet and differentiated in alcohol.—*T. E. Weier.*

MICROORGANISMS

HAITINGER, M., and SCHWERTNER, R. **Beiträge zur Fluoreszenzmikroskopie in der Bakteriologie.** *Zentbl. Bakt. I Abt. Orig.*, **145**, 141–4. 1940.

Smears of sputum and pus gave satisfactory results with the fluorescent microscope when the following technic was used:

Fix smears with heat; flood with 1:500 aqueous acridin for 30 sec.; wash quickly; differentiate 10–15 sec. with ethyl alcohol containing 4% HCl; wash and dry in air or with heat. Bacteria stain a golden color in a midnight-blue field.—*H. J. Peppler.*

HANNA, G. D., and GRANT, W. M. **Preliminary note on a technique for mounting diatoms in realgar and other substances.** *J. Roy. Micr. Soc.*, **59**, 174–6. 1939.

Exceedingly stable films of realgar, selenium, or tellurium can be produced by evaporation in a vacuum. The same is true of arsenic, which however seems to adhere less tightly. Standard apparatus used in the aluminizing of mirrors for microscope illuminators is employed; the tungsten filament commonly used for aluminum is replaced by a short band of nichrome ribbon. A minute amount of the material to be evaporated is placed on a thin sheet of mica, which is rested on the heater. The cover slips bearing strewn diatoms are attached to a lantern-slide cover plate, and this is suspended, diatoms facing downward, a few inches above the heater. Evaporation requires but a few seconds. Covers so treated are attached to slides with hyrax and surrounded by rings of shellac. Resolution is as satisfactory if the diatoms are coated with a half wave-length of the material as if the thickness equals two or three wave-lengths.—*C. E. Allen.*

HOLLBORN, K. **Ein verbesserter Gram-Simultanfarbstoff.** *Zentbl. Bakt., I Abt. Orig.*, **143**, 160. 1939.

The victoria-blue-pyronin technic for Gram's staining procedure (*Zentbl. Bakt. I Abt. Orig.*, **136**, 506, 1936) has been improved and now can be applied to smears in routine examination for gonococci. The following procedure has been suggested: flood extremely thin smears of pus with the staining solution for 1 min.; wash in distilled water; blot with filter paper; dry in air. The dye mixture for the simultaneous Gram procedure is obtainable only from the author (K. Hollborn and Söhne. Leipzig).—*H. J. Peppler.*

KNAYSI, GEORGES. **The demonstration of a nucleus in the cell of a staphylococcus.** *J. Bact.*, **43**, 365–85. 1942.

Several chemical reactions have been adapted to demonstrate the nuclear nature of a central granule in a staphylococcus. These are as follows:

1. Stain dried flame-fixed smears with methylene blue solutions of varying pH (to 5 ml. of 1% KH_2PO_4 add 0.1 ml. distilled water, 0.1 ml. *N* HCl, or 0.2 ml. *N* HCl and then 0.1 ml. 1% aqueous methylene blue, to each) while observing

under the microscope. The nucleus stains more deeply than the cytoplasm at the lower pH values. It may be necessary to decolorize with 1% H_2SO_4 to see the nucleus of a rapidly growing cell.

2. Stain dried flame-fixed smear with Meyer's methylene blue (1 ml. saturated alcoholic methylene blue diluted with 10 ml. of water) for 2 min.; drain the slide and place it in 1% aqueous methylene blue containing 1 or 2% acetic acid for 3 min.; rinse thoroly in running water; blot; dry; and examine.

3. Millon's test for proteins. Flood the slide with Millon's reagent (1 ml. mercury in 9 ml. HNO_3, sp. gr. 1.420 to 1.425, diluted with twice as much water) and heat until steam is visible several times; rinse with running water; remove the white precipitate by immersing the slide in 1% HNO_3; rinse again; blot; dry; examine in a drop of water under a cover glass. The nuclear protein gives a reddish brown color.

4. Sharp's test for proteins. Place the air-dried flame-fixed smear in 5% glucose at 100° C. for 24 hr.; rinse thoroly in running water; dry; examine for dark brown protein particles which appear to be stained with colloidal silver; place a drop of 10% NaOH on the smear under the cover glass and seal it in place; the color is removed slowly.—*Virgene Kavanagh.*

LIND, HOWARD E., and SHAUGHNESSY, HOWARD J. **Fluorescent staining technic for the detection of acid fast bacilli.** *J. Lab. and Clin. Med.*, **27**, 531–6. 1942.

The flourescent technic used was as follows: Apply auramin solution (0.1 g. auramin, National Aniline Co., and 57 cc. distilled water) for 2 min. Wash and decolorize (70% alcohol 100 cc., concentrated HCl 0.5 cc., and NaCl 0.5 g.) for 2–4 min.; then decolorize for 2 min. more in fresh decolorizing solution. Wash, dry and examine. The necessary special equipment may be obtained from the Spencer Lens Co. Place the blue ultraviolet filter on the lamp in front of the light, replace the mirror by one coated with aluminum and focus as much light as possible into the draw tube. An 8 mm. objective and 20× ocular are recommended. This gives a field 20 times that of the usual oil immersion field, with a magnification of 400. The acid-fast bacilli appear as small, bright yellow rods against a dark background. The fluorescent method proved to be more sensitive than the Ziehl-Neelsen technic even tho the specimens of sputum being examined contained tricresol which produces fluorescence and interferes with the examination. The disadvantages of the fluorescent technic are the use of a monocular microscope, the interference by tricresol, and possible greater fatigue. These are counterbalanced by the advantages of larger fields, simplicity, rapidity, and increase in the number of positives.—*John T. Myers.*

MANER, R. **Über die Färbung der Spirochaeta icterohaemorrhagiae und anderer Leptospiren.** *Zentbl. Bakt., I Abt. Orig.*, **143**, 462–4. 1939.

Twenty different dyes commonly used in staining parasites failed to reveal selected spirochaetes as colored or colorless bodies. Smears fixed with methyl and ethyl alcohol, or a mixture of alcohol and ether (no ratio given), or osmium vapor and stained with Giemsa's solution were most satisfactory for practical purposes.—*H. J. Peppler.*

NERI, F. **Beobachtungen über den Geiszelapparat der Bakterien (mit einer neuen Färbungsmethode der Bakteriengeiszeln).** *Zentbl. Bakt., I Abt. Orig.*, **146**, 166–74. 1940.

The curled flagella of certain strains of *Salmonella typhi-suis* were best demonstrated by the following method:

Fixing and mordant: (a) saturated $HgCl_2$ (10–15% hot solution); (b) 20% tannic acid + 37 ml. saturated $KAl(SO_4)_2+12H_2O$ (15% hot solution filtered after cooling); (c) 10% $FeNH_4(SO_4)_2+12H_2O$ in cold distilled water. Mix a, b, c (2:1:1); filter before use. (The mixture is good for 2–3 hr.)

Place air-dry slides of 18–24 hr. culture inverted in a watch glass; add mordant until slide floats, and allow to stand for 1 hr.; rinse with distilled water; stain (0.5 g. crystal violet + 10 ml. ethyl alcohol + 90 ml. anilin water) several minutes with heat.—*H. J. Peppler.*

SERLIN, N. J., and LISA, J. R. **A concentration technic for gametocytes of estivo-autumnal malaria.** *Amer. J. Clin. Path., Tech. Suppl.*, **6**, 8. 1942.

The following method, based on the fact that erythrocytes containing plasmodia have a lower specific gravity than normal red cells, is recommended for examination of blood containing only gametocytes: Evaporate completely 1 ml. of 1% aqueous potassium oxalate in a 15 cc. centrifuge tube; add 5 ml. blood; mix carefully; centrifuge ½ hr. at 2,500 R/M; discard all but ⅛ in. of supernatant plasma; apply one drop of the buffy layer to a glass slide and spread; stain by Wright's method.—*H. Gordon.*

STEUER, W., and SCHINDLER, E. **Über die Leistungsfähigkeit des Brilliantgrünphenolrotagars zur Typhusparatyphusdiagnose.** *Zentbl. Bakt., I Abt. Orig.*, **146**, 49–53. 1940.

A comparative study of the usefulness of Endo agar and brilliant-green-phenol-red agar for the identification of *Salmonella sp.* and *Eberthella sp.* in stools and urine was made with 4053 samples. The brilliant green medium was distinctly superior to Endo agar, showing a marked inhibition of *Proteus sp.*, *Escherichia coli*, and *Shigella sp.* Numerous factors were found to influence the effectiveness of the medium. The following method of preparation of brilliant-green-phenol-red agar is recommended: beef infusion (quantity not stated), 1% peptone, 0.5% NaCl, 1.5% lactose, 4.0 ml. of 0.2% phenol red (to the liter?), 1.0 ml. of 0.5% aqueous brilliant green (to the liter?), 2.5% agar; reaction adjusted to pH 6.8.—*H. J. Peppler.*

WEISS, CHARLES. **An improved technic for the isolation of Bact. granulosis (Noguchia granulosis).** *Amer. J. Clin. Path., Tech. Suppl.*, **6**, 19–21. 1942.

Pure cultures of *N. granulosis* may be isolated by the following method: Dilute 1 part Noguchi's leptospira semi-solid medium with 2 parts water; mix 0.5 ml. with an equal volume of 1:5000 aqueous gentian violet or Victoria blue; inoculate medium with 0.02 ml. of suspension; incubate 15 min.; streak on Noguchi's solid leptospira medium; incubate 1 week or longer. The dye inhibits the growth of staphylococci, without affecting *N. granulosis.*—*H. Gordon.*